IMMUNOCHEMICAL STUDIES ON METHYLGLYOXAL MODIFIED HUMAN DNA: ROLE IN DIABETES MELLITUS

ABSTRACT

OF THE

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

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BY

SAHEEM AHMAD

DEPARTMENT OF BIOCHEMISTRY
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ALIGARH (INDIA)

2010

Abstract

The highly reactive electrophile, Methylglyoxal (MG), a break down product of carbohydrate is a major environmental mutagen and has potential genotoxic effects. It is ubiquitous in beverages and foods, such as coffee, toast and soy sauce, as well as in cigarette smoke. Its mutagenicity has also been reported in *Escherichia coli*, wherein it causes mutations at G:C base pairs, as well as in *Sacharomyces cerevisiae*. Previous studies have suggested that MG reacts with free amino groups of proteins and DNA under physiological conditions and forms advanced glycation end products resulting in the generation of hydroxyl ($^{\cdot}OH$) and superoxide ($O_2^{\cdot-}$) ions which play an important role in the pathophysiology of ageing and diabetic complications. Moreover, in glycation reaction the free carbonyl groups of the sugar react with the free amino residues of the macromolecules in a series of chemical processes known as Maillard reaction. Initiation of glycation occurs by the formation of acid-labile Schiff base adducts which undergoes Amadori or Heyn's rearrangements into more stable products. Glycated DNA has attracted much attention in the past few years and is considered to be a pathogenic factor for diabetes mellitus. During the disorder in the metabolism of reducing sugars, the sugar molecules can initiate glycation of DNA *in vivo*. Previous investigations by several scientists using biochemical and molecular biological methods have shown that DNA structure and function are affected by the addition of sugars and carbonyls (MG), resulting in deleterious modifications and other mutations.

Diabetes mellitus refers to group of common metabolic disorders that share the phenotype of hyperglycemia. Several types of diabetes mellitus exist and are caused by a complex interaction of genetics and environmental factors and viral infection. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications. Mechanisms by which increased oxidative stress is involved in the diabetic complications are partly known, including activation of transcription factors, advanced glycated end products (AGEs) and protein kinase C. In diabetes mellitus, low insulin levels prevent cells from absorbing glucose; as a result glucose builds up in the blood. Absolute insulin deficiency caused by autoimmune-mediated destruction of pancreatic β-cells characterizes type I diabetes. The main cause of the beta cell loss is a T-cell mediated immune attack. There is a strong association of type I diabetes with individuals who possess particular HLA haplotypes. Viruses, such as enteroviruses, coxackie virus and

rubella, have been proposed as culprits but have not been definitively shown to induce type I diabetes. None of these theories has evolved into a clear cause-and-effect initiator of diabetes. By whatever initiating mechanism, the autoimmune destruction of β-cells leads to a progressive decline in the body's insulin secretory capacity.

Like type I diabetes, type II diabetes also involves both genetic susceptibility and environmental factors. It is caused by a combination of insulin resistance and relative insulin deficiency with increased hepatic glucose production. Insulin resistance is generally thought to precede insulin deficiency. Before type II diabetes develops, the pancreatic β-cells increase their production of insulin to compensate for increased insulin resistance. For unclear reasons, β-cell secretory capacity gradually declines in some individuals, leading to the development of type II diabetes. The cause of β-cell failure in type II diabetes is unknown.

In the present study, commercially available human DNA was modified by MG and lysine in the presence of Cu^{2+}. DNA modifications were analyzed by various spectroscopic and analytical techniques like, UV, Fluorescence, CD, thermal denaturation studies, HPLC, NMR, LC-MS and ESI-MS. Moreover, agarose gel electrophoresis, nuclease S1 digestibility and comet assays were also performed to assess the modification of the MG-Lys-Cu^{2+} system. Furthermore, antibodies were induced in rabbits against native and modified DNA. The induced antibodies were characterized with respect to antigen binding specificities by direct binding and inhibition ELISA. Antigen binding specificities were further confirmed by band shift assay. These antibodies have been used as an immunochemical probe to detect reactive carbonyl species (RCS) induced DNA damage in diabetes patients. In order to assess the possible role of MG-Lys-Cu^{2+} modified DNA in the etiology of type I diabetes mellitus, sera from patients were assessed for their binding to native and MG-Lys-Cu^{2+} modified human DNA. Furthermore, binding of serum antibodies for type II diabetes patients with native and MG-Lys-Cu^{2+} modified human DNA was also evaluated.

The UV & CD spectroscopical analysis, agarose gel electrophoresis, nuclease S1 digestibility assay and thermal denaturation studies, suggest structural perturbation in DNA as a result of modification. This might be due to the generation of single-stranded regions, destabilization of hydrogen bonds and modification of nitrogenous

bases which result in the destruction of chromophoric groups through attack on the sugar-phosphate back bone. The fluorescence spectroscopy study suggests formation of advanced glycation end-products. This is further proved by anti-glycation study and UV- spectroscopy. The genotoxicity of MG-Lys-Cu^{2+} formed AGEs was confirmed by using the comet-assay as an endpoint for DNA damage. This is evident from the DNA breakage and subsequent formation of the comet tail. Moreover, the adduct (CEdG) formed due to the glycation reaction of MG-Lys-Cu^{2+} with human DNA was detected by HPLC, supported by NMR and further confirmed by LC-MS.

The reaction of MG and lysine generates free radicals which were confirmed by quantitation of hydroxyl and superoxide radicals. The generation of these free radicals was further proved by quenching studies. Therefore, results presented here indicate that the glycation reaction of MG with lysine in the presence of Cu^{2+} may lead to oxidative damage of DNA through a mechanism that involves hydroxyl radicals.

The MG-Lys-Cu^{2+} modified human DNA proved to be a potent antigen, eliciting high titre immunogen specific antibodies in rabbits. The antigenic specificity of anti-MG-Lys-Cu^{2+} modified human DNA IgG was ascertained by competitive binding assay. A maximum of 88.5% inhibition in the antibody activity at inhibitor (immunogen) concentration of 20 μg/ml and just 2.8 μg/ml of the inhibitor concentration caused 50% inhibition, clearly indicating very high specificity and affinity of the induced antibodies towards the immunogen, i.e. the MG-Lys-Cu^{2+} modified human DNA. Affinity purified immune IgG showed higher specificity as compared to serum. Moreover, visual detection of interaction between immune IgG and the immunogen was done by band shift assay. The result shows high affinity of the induced antibodies for the immunogen. The induced antibodies though highly specific for MG-Lys-Cu^{2+} modified human DNA also exhibited polyspecificity, recognizing various nucleic acid conformers and nitrogenous bases in competitive inhibition assay. The results suggest that MG modification of DNA in presence of lysine and Cu^{2+} caused structural perturbations generating new epitopes thus transforming it into a potential immunogen. The modified DNA may be one of the factors for the induction of circulating anti-DNA antibodies in diabetes.

The clinical study focuses on the possible involvement of MG-Lys-Cu^{2+}-modified and the unmodified human DNA in diabetes mellitus (both type I & II) was probed. Out of 40 sera in type I diabetes, 67.5% showed preferentially high binding to MG-Lys-Cu^{2+} modified human DNA as compared to its native analogue. These results indicate substantial recognition of MG-Lys-Cu^{2+} modified human DNA by the auto-antibodies in diabetic (type-I) patients. The affinity purified IgG from diabetic patients showed appreciably high binding towards MG-Lys-Cu^{2+} modified human DNA, reiterating the results obtained with serum antibodies. The strong binding of auto-antibodies from diabetes type I patients to MG-Lys-Cu^{2+} modified human DNA is evidence towards the involvement of modified bases and single strand regions in disease pathogenesis. The spontaneous production of auto-antibodies in type I diabetes might be a result of the generation of the antigenic epitopes on the DNA molecules that are recognized as 'non self' by the body's immune system. It could, therefore, be one of the factors of the immune response in diabetes. In type II diabetes, out of 45 sera only 35.5% showed low to moderate binding to MG-Lys-Cu^{2+} modified human DNA as compared to its native analogue. In type II diabetes, the recognition of auto-antibodies against MG-Lys-Cu^{2+} modified human DNA is quite low as compared to type I diabetes. The results show ample evidence of the involvement of MG-Lys-Cu^{2+} modified human DNA in type I diabetes while the same is less established for the type II.

In view of all the above studies it could be concluded that MG-Lys-Cu^{2+} modification of human DNA resulted in the formation of single strand breaks and base modification causing perturbation in the structure of DNA. Moreover, modified human DNA was highly immunogenic in experimental animals. The induced antibodies, though highly specific for the immunogen, also exhibited polyspecific binding. The antibodies showed significant binding with various nucleic acid conformers and nitrogenous bases. Higher recognition of MG-Lys-Cu^{2+} modified human DNA by diabetes type I autoantibodies is a clear indication of MG-Lys-Cu^{2+} induced DNA damage in these patients. It could, therefore, be one of the factors for the autoimmune response leading to the induction of circulating anti-DNA *autoantibodies in diabetes mellitus, type I. Furthermore, antibodies from type II* diabetes patients exhibited low to moderate binding with MG-Lys-Cu^{2+} modified human DNA.

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Dated : .....

Approved :-.....

.....

.....

Dr. Moinuddin (*Supervisor*)

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ALIGARH (INDIA)

2010

Dedicated

To

My Abbu

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DEPARTMENT OF BIOCHEMISTRY
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Certificate

I certify that the work presented in the thesis entitled **"Immunochemical studies on methylglyoxal modified human DNA: role in diabetes mellitus"** has been carried out by **Mr. Saheem Ahmad**, in the Department of Biochemistry, under my direct supervision and is suitable for the award of Ph.D degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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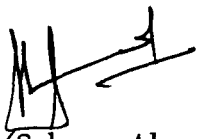
"Don't be worried about losing the battle if this helps u win the war"



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(Saheem Ahmad)

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In view of all the above studies it could be concluded that MG-Lys-Cu^{2+} modification of human DNA resulted in the formation of single strand breaks and base modification causing perturbation in the structure of DNA. Moreover, modified human DNA was highly immunogenic in experimental animals. The induced antibodies, though highly specific for the immunogen, also exhibited polyspecific binding. The antibodies showed significant binding with various nucleic acid conformers and nitrogenous bases. Higher recognition of MG-Lys-Cu^{2+} modified human DNA by diabetes type I autoantibodies is a clear indication of MG-Lys-Cu^{2+} induced DNA damage in these patients. It could, therefore, be one of the factors for the autoimmune response leading to the induction of circulating anti-DNA autoantibodies in diabetes mellitus, type I. Furthermore, antibodies from type II diabetes patients exhibited low to moderate binding with MG-Lys-Cu^{2+} modified human DNA.

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Abbreviations

A_{260}	: Absorbance at 260 nm
A_{280}	: Absorbance at 280 nm
AGEs	: Advanced glycation end products
APS	: Ammonium persulphate
bp	: Base pair
BSA	: Bovine serum albumin
CT DNA	: Calf thymus deoxyribonucleic acid
CEdG	: Carboxyethyl-2′-deoxy guanosine
DNA	: Deoxyribonucleic acid
DHAP	: Dihydroxyacetone phosphate
DMSO	: Dimethyl sulfoxide
DTPA	: Diethylenetriamine pentacetic acid
EDTA	: Ethylene diamine tetracetic acid
ELISA	: Enzyme linked immunosorbent assay
gm	: Gram
G3P	: Glyceraldehyde -3-phosphate
hr	: Hour
H_2O_2	: Hydrogen peroxide
$^{\cdot}OH$	: Hydroxyl radical
HPLC	: High performance liquid chromatography
IgG	: Immunoglobulin G
L	: Litre
Lys	: Lysine
µL	: Microlitre
M	: Molar
mBSA	: Methylated bovine serum albumin
MG	: Methylglyoxal
NO	: Nitric oxide
nM	: Nanometer
NHS	: Normal human sera
nDNA	: Native DNA

$O_2^{\cdot-}$	: Superoxide anion radical
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffer saline
RCS	: Reactive carbonyl species
ROS	: Reactive oxygen species
SSC	: Saline sodium citrate
SDS	: Sodium dodecyl sulphate
SOD	: Superoxide dismutase
Tris	: Tris (hydroxymethyl) amino methane
TBS-T	: Tris buffer saline with 0.05% Tween-20
Tm	: Melting temperature
TEMED	: N,N,N',N'-tetraethylmethylene diamine
UV	: Ultraviolet
λ_{max}	: Wavelength maxima
λ_{min}	: Wavelength minima

Introduction

In 1953, the discovery of the structure of DNA was landmark in the history of DNA biology which sheds light on the inheritance of genetic material, replication, DNA damage, repair, diversity and the evolution of species. Its discovery has united genetics with biochemistry, free radical biology, medicine and physiology. Briefly, the model proposed by James Watson and Francis Crick is based on two paired DNA strands that are complementary in their nucleotide sequence. The model has striking implications for the process of DNA replication and recombination. The DNA in a fully hydrated medium is found in B form (Langridge *et al.*, 1960) and this is the most accepted structure of DNA molecule in solution. X-ray diffraction studies have shown that a number of variations in conformation of this basic structure can occur (Adams *et al.*, 1981). It is well known that the right handed double helical DNA can exist in A, B, C and D conformations, whereas the left handed polynucleotide can adopt the Z structure (Saenger, 1984), so called because phosphate groups in the backbone are zig-zagged (Rich *et al.*, 1984). The importance of DNA lies in its unique property to adopt multiple conformations depending on its bioenvironment. The DNA double helix is both deformed and made deformable by its local base sequence, whereas other regions may be made especially susceptible to a change in conformation when the helix interacts with protein, or an adjacent DNA helix in a crystal lattice.

Antigenicity of native and modified DNA

Anti-DNA autoantibodies permanently attract the attention of researchers. In spite of numerous investigations aimed to determine their potential targets and role in the disease, origins of anti-DNA autoantibodies and their pathological role remain to be established. Moreover, taking into account of such newly described features of anti-DNA antibodies as the ability to penetrate into the living cell (Alarcon *et al.*, 1996) and DNA-hydrolyzing activity (Schuster *et al.*, 1992), one may under estimate pathogenic potential of DNA-specific autoantibodies. In general, antibodies that bind DNA do not display strict disease specificity. It is, however, widely accepted that healthy individuals usually express low-affinity antibodies with specificity to single-stranded DNA, while presumably pathogenic high-affinity autoantibodies to double-stranded DNA are frequently overrepresented on the background of systemic autoimmune abnormalities (Paul *et al.*, 1990) and their presence appears a diagnostic criterion for SLE (Bootsma *et al.*, 1996). Some blood tumours and even AIDS are

also characterized by the increase in production of DNA-specific antibodies (Hamblin *et al.*, 1986; Rodriguez *et al.*, 1994). The major known pathogenic process induced by anti-DNA antibodies is the inflammatory response due to their deposition in kidney in form of the immune complexes (Suenanga *et al.*, 1996). However, DNA specific autoantibodies can also trigger apoptosis in cultured mesangial and endothelial cells (Tsai *et al.*, 1993; Lai *et al.*, 1997) and are cytotoxic to the primary cultures of lymphocytes (Shoenfeld *et al.*, 1985). Non-immunogenic status of native DNA has led to the assumption that natural anti-DNA antibodies may be formed against DNA–protein complexes, such as nucleosomes, persisting in blood as a result of disease mediated increase in cellular destruction (Jacob *et al.*, 1992; Mohan *et al.*, 1993). Cross reactivity with proteinaceous nuclear antigens, displayed by anti-DNA antibodies, contributed to this hypothesis and even allowed to specify a pool of anti-nuclear antibodies (ANA) (Tan *et al.*, 1988). Described cross-reactivity of anti-DNA antibodies with membrane antigens (Raz *et al.*, 1993) may potentially result in triggering of certain cellular responses through signal transduction pathways. Polyspecificity of anti-DNA antibodies was also shown to favor to their capacity of penetration into living cell (Avrameas *et al.*, 1998). Entry of anti-DNA autoantibodies into living cell is shown to be accompanied by certain cellular dysfunctions (Tsai *et al.*, 1993; Lai *et al.*, 1997; Yu *et al.*, 1998). In some cases, penetration of antibodies into the cells is connected with either induction (Tsai *et al.*, 1993) or suppression of apoptosis (Yanase *et al.*, 1997), however, precise consequences of antibody entry into the cell and its pathogenic significance remain to be determined. Catalytic activity of anti-DNA autoantibodies (DNA-abzymes) *in vitro* was described in a number of studies (Schuster *et al.*, 1992; Gololobov *et al.*, 1997), however, physiological essence of antibody DNA-hydrolyzing activity remains elusive. It is evident, that any detrimental effect to the cell that the DNA-abzyme may cause, requires direct contact between DNA-hydrolyzing autoantibodies and chromatin of the living cell.

In 1957, anti-DNA antibodies was identified as a biomarker for systemic lupus erythematosus (SLE) autoantibodies, since then antigenic property of nucleic acids received much attention (Cepellini *et al.*, 1957; Meischer and Strassie, 1957; Robbins et al., 1957). DNA is a complex molecule whose immunologic properties vary with base sequence and encompass both stimulation and inhibition (Messina *et al.*, 1991;

Krieg *et al.*, 1998; Pisetsky and Reich, 2000). The problem of how an individual can immunologically distinguish between self and non self has fascinated and perplexed immunologists. When this distinction cannot be made, autoimmune diseases such as systemic lupus erythematosus (SLE) occur and are associated with the appearance of large amounts of autoantibodies. In the case of lupus, antibodies that react with double-stranded DNA are one of the hallmarks of the disease (Kofler, 1984). In the early 1960s, methods were developed for the experimental induction of antibodies to nucleic acids (Levine *et al.*, 1960; Erlanger and Beiser, 1964; Plescia *et al.*, 1964; Halloran and Parker, 1966) which until then had been considered widely to be non-immunogenic. Studies on the antigenic specificity of anti-DNA antibodies have enforced the view that DNA is immunologically 'simple and bland'. It has been well established that native DNA in B-conformation is not immunogenic. Nevertheless, various modified forms of DNA, DNA in complexes with DNA-binding proteins are immunogenic and induce antibodies. Origin of anti-ds DNA autoantibodies in cancer patients and tumour-bearing mice has been demonstrated (Zhang *et al.*, 2002). Exogenous native B-DNA has not been found immunogenic in experimental animals. Immunization of experimental animals with denatured DNA, synthetic nucleic acid polymers like poly (dT), poly (dC), poly (dA), poly (A), poly (I), poly (G), dsRNA, left handed Z-DNA, chemically modified DNA and certain helical synthetic polynucleotides with the exception of native B-DNA, induced antibodies that react selectively with the immunogen and do not cross react with native DNA (Stollar, 1986; Anderson *et al.*, 1988).

Mammalian DNA elicit poor responses to single stranded DNA and fails to induce antibodies against native or double stranded DNA, the serologic hallmark of SLE (Madaio *et al.*, 1984). However, it has been shown that DNA complexes with synthetic peptide FUS-I can induce anti-dsDNA response in mice (Desai *et al.*, 1993). Since the antibody reactivity to both single and double stranded form appears independent of DNA species origin, these finding suggest recognition of conserved conformational determinants i.e., helical backbone of B-DNA and a lack of DNA sequence micro heterogeneity on antigenicity (Stollar, 1975; Pisetsky, 1993). Anti-DNA antibodies may also result by autoimmunization with chromatin, rather than native DNA (Theofilopoulos, 1995). The left handed Z-DNA is an example of a helical DNA that is a much more potent immunogen than native B-helical DNA

(Lafer *et al.*, 1981; Madaio *et al.*, 1984). The antibodies induced by Z-DNA have high degree of selectivity and specificity as they react with immunogen but not with B-DNA or ssDNA. The DNA modified by either chemically or physically that differ significantly from B-DNA are much stronger immunologic stimuli than nDNA and most of the antibodies induced by modified nDNA do not react with unmodified DNA (Anderson *et al.*, 1988). Polynucleotides in B-conformation acquired immunogenicity after modification with furocouramin (Arif and Ali, 1996). DNA modified with drugs, hormones, free radicals etc, has been reported to induce antibodies against the immunogen (Moinuddin and ali, 1994; Dixit *et al*, 2005; Habib *et al.*, 2005; Khan *et al.*, 2006). Recently, the role of peroxynitrite ($ONOO^-$) modified human placental DNA in the induction of circulating cancer autoantibodies has been reported (Habib *et al.*, 2009).

Glycation of biomacromolecules

Glycation is the nonenzymatic addition of reducing sugars (glucose, fructose, mannose, etc. and their phosphate derivatives) as well as compounds related to sugars (e.g. ascorbic acid, methylglyoxal (MG), glyoxal and 3-deoxyglucosone etc.) into biological macromolecules (proteins, DNA and lipids) (Bucala, 1985; Suarez, 1989; Sengupta, 2005). The free carbonyl groups (-C=O) of the sugar and related moieties react with the free amino ($-NH_2$) residues of the macromolecules in a series of chemical processes known as Maillard reaction. Initiation of glycation occurs by the formation of acid-labile Schiff base adducts which undergoes Amadori or Heyn's rearrangements into more stable products. The early glycation products undergo slow transformation to yield the irreversible advanced glycation end products (AGEs). These reactions have attracted significant attention in recent days because of their association with the production of free radicals, which play roles in the development of cancer, diabetes, heart disease, cataract, atherosclerosis and neurodegenerative disorder (e.g., Parkinson's disease, Alzheimer's disease), etc.

The products of non enzymatic glycation and oxidation of proteins, DNA and lipids, the advanced glycation end products (AGEs), accumulate in a wide variety of environments. AGEs may be generated rapidly or over long times stimulated by a range of distinct triggering mechanisms, thereby accounting for their roles in multiple settings and disease states. A critical property of AGEs is their ability to activate

receptor for advanced glycation end products (RAGE), a signal transduction receptor of the immunoglobulin superfamily. Due to such interaction, AGEs impart a potent impact in tissues, stimulating processes linked to inflammation and its consequences. Therefore, AGEs cause perturbation in a diverse group of diseases, such as diabetes, inflammation, neurodegeneration and aging (Ramasamy *et al.*, 2005).

A large body of evidence suggests that AGE formation and accumulation are enhanced in diabetes (Brownlee, 1992). AGEs are a heterogeneous class of compounds that are composed of both fluorescent and non-fluorescent species. Glycation adducts of proteins are formed when proteins react with glucose-reactive alpha-oxoaldehydes such as glyoxal, methylglyoxal and 3-deoxyglucosone (3-DG) (Brownlee, 1996). The initial Schiff base adducts formed from glucose and lysine and N-terminal amino acid residues may rearrange to form the key intermediate, fructosamine. Fructosamine degradation and the direct reaction of alpha oxoaldehydes with proteins may form many AGEs. Of the various types of AGEs that may be generated, it has been shown that both cross-linked and noncross-linked AGEs may be generated. *In vivo*, a diverse array of AGE products has been detected and characterized such as bis(lysyl) imidazolium cross-links, hydroimidazolones and monolysyl adducts (Wautier and Schmidt, 2004). It has been shown that both carboxymethyl lysine (CML) adducts of proteins or lipids, as well as AGEs derived through the generation of hydroimidazolone, species that accumulate in diabetes, are specific ligands for RAGE (Thornalley, 1998; Kislinger *et al.*, 1999). Indeed, evidence indicates that CML-AGEs are highly prevalent in diabetes, as well as in aging and renal failure (Reddy *et al.*, 1995; Ikeda *et al.*, 1996; Schleicher *et al.*, 1997; Tauer *et al.*, 2001).

DNA glycation and AGEs

Glycated DNA has attracted much attention in the past few years and is considered to be a pathogenic factor for diabetes mellitus. During the disorder in the metabolism of reducing sugars, the sugar molecules can initiate glycation of DNA *in vivo* (Lee and Cerami, 1989; Levi and Werman, 2003; Dutta *et al.*, 2005). Previous investigations by several scientists (Bucala *et al.*, 1985; Lee, 1989; Dutta *et al.*, 2005) using biochemical and molecular biological methods have shown that DNA structure

and function are affected by the addition of sugars, resulting in deleterious modifications and other mutations.

Advanced glycation end-products (AGEs) of DNA are formed spontaneously by the reaction of carbonyl compounds such as sugars, methylglyoxal or dihydroxyacetone *in vitro* and *in vivo*. After a complex cascade of dehydration, condensation, fragmentation, oxidation and cyclization reactions, a diverse and largely undefined group of compounds termed AGEs are formed (Schleicher *et al.*, 2001). Only few AGEs have been characterized chemically and identified in tissues, the most investigated of which has been carboxymethyllysine (CML). In renal disease AGE accumulation is ascribed both to the impaired elimination of AGEs and to enhanced formation due to oxidative stress (Heidland *et al.*, 2001). Accumulation of AGEs in tissues is a common phenomenon of normal aging and occurs at accelerated rates in patients with diabetes mellitus (Schleicher *et al.*, 1997; Baynes, 2001). In *in vitro* studies, mutagenic effects of DNA-AGE such as deletions, insertions and transposon activation were shown in bacterial model systems (Pischetsrieder *et al.*, 1999). Furthermore, it is shown that DNA can be glycated *in vitro* yielding carboxyethylguanosine as major products. However, little is known about the biological consequences of DNA-AGEs.

In vitro, nucleobases and dsDNA react with sugars in a similar way as proteins (Lee and Cerami, 1987; Knerr and Severin, 1993; Singh *et al.*, 2001). The exocyclic amino group of 2'-deoxyguanosine is particularly prone to glycation reactions, leading to the formation of N^2-carboxyethyl, N^2-carboxymethyl, N^2-(1-carboxy-3-hydroxypropyl), and N^2-(1-carboxy-3,4,5-trihydroxypentyl) modifications, as well as cyclic dicarbonyl adducts (Ochs and Severin, 1994). The two diastereomers of N^2-carboxyethyl- 2'-deoxyguanosine (CEdG $_{A,B}$) are stable reaction products that are formed from a variety of glycating agents, such as glucose, ascorbic acid, glyceraldehyde, dihydroxyacetone (DHA), or methylglyoxal (Larisch *et al.*, 1998; Frischmann *et al.*, 2005). Recently, carboxyethylated nucleobases were detected in human urine (Schneider *et al.*, 2004) indicating the formation of DNA AGEs in the healthy human organism. A significantly increased number of CEdG positive cells were immunostained in glomeruli of patients with diabetic nephropathy as compared to healthy controls (Li *et al.*, 2006) as well as in glomeruli of diabetic rats (Nakamura

et al., 2007). DNA AGEs are potentially genotoxic compounds because they induce depurination as well as single strand breaks and lead to mutations (Pischetsrieder, 1999) *in vitro*. *In vivo*, it was shown, for example, that 3-deoxyglucosone, a glucose degradation product, induces embryonic malformation and teratogenicity, effects that may be related to DNA AGEs (Eriksson *et al.*, 1998). DNA glycation in cultured cells was observed using radioactively labeled glucose (Shires *et al.*, 1990) or a ^{32}P-postlabeling technique (Vaca *et al.*, 1998). Furthermore, the presence of $CEdG_{A,B}$ was detected in cultured cells by HPLC–diode array detector (DAD) after immunoaffinity chromatography (Schneider *et al.*, 2006).

Formation of AGEs

Maillard first described the non-enzymatic reaction of glycine with glucose in 1912 (Maillard, 1912). The reducing sugars and other α-dicarbonyl compounds (glyoxal, MG and 3-deoxyglucosone) form a range of heterogeneous AGE adducts on free amine and thiol groups within proteins, lipoproteins and nucleic acids. These adducts alter the structure and function of their target molecules but also form ligands for the receptor for AGEs, RAGE (Nass *et al.*, 2007) and other known AGE receptors (Ahmed and Thornalley, 2007). Structural and inflammatory damage by AGEs is implicated in propagation of diabetes vascular and neuronal complications. MG is a potent AGE precursor, forming adducts on arginine (Arg), lysine (Lys), cysteine (Cys) and deoxyguanosine (dG) residues. The most prevalent MG adduct in proteins is the MG-derived hydroimidazolone (MG-H), but carboxyethyl lysine (CEL), Nδ- (4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)-ornithine) and argpyrimidine are also formed, as well as the crosslinking dimer MOLD (methylglyoxal lysine dimer). MG-H and CEL equivalents are also found on dG residues within DNA (dG-MG and CEdG), and MG forms a hemithioacetal with thiols, such as that in Cys. MG-AGEs are common in human tissues and are known to play critical roles in diabetes vascular complications (Thornalley, 2008). MG-H is thought to be a ligand for RAGE (Thornalley, 1998), but the role of MG-AGEs in other AGE receptor systems is unclear.

Detection of AGEs

AGE detection can be based on the fluorescent properties of AGEs (Monnier and Cerami, 1981). After the excitation at 370 nm, fluorescence emission wavelength is

typically at 440 nm due to the presence of heterocyclic compounds. However, the exact quantitation of AGEs is difficult to achieve due to the lack of proper means to represent the whole diverse AGE family. Candiano *et al.* (1986) have suggested an alternative method for quantitation involving the formation of a chromophore after reacting AGEs with diazonium salts and subsequently measuring absorbance at 490 nm wavelength. Recently, the use of immunoassays has been incorporated into many AGE investigations, including research on pyrraline (Miyata and Monnier, 1992), lipoprotein AGEs (Doucet *et al.*, 1995), CML (Reddy *et al.*, 1995), pentosidine (Miyata *et al.*, 1996), ribonuclease AGEs, AGE crosslinks (Vasan *et al.*, 1996) and imidazolones (Niwa *et al.*, 1997). However, the antibodies produced may recognize only a limited number of AGEs and leave others undetected.

Reactive carbonyl species

Reactive carbonyl species (RCS) are potent mediators of cellular carbonyl stress originating from endogenous chemical processes such as lipid peroxidation and glycation. RCS are a heterogeneous group of small molecular weight carbonyls activated by α, β-unsaturation as in 4-hydroxynonenal and acrolein, α-oxo-substitution as in glyoxal and methylglyoxal, an β-oxo-substitution as in malondialdehyde (Thornalley, 1996). DNA adducts formed by RCS are known to possess strong miscoding potential *in vitro* and of the DNA adducts formed by MDA, pyrimido(1,2α) purin-10(3H) one (M_1G) is readily detected in many human tissues. Adducts formed by HNE have been detected in healthy human colon tissue (Wacker *et al.*, 2000). Etheno-DNA adducts are formed from the epoxides of enals and elevated levels of this type reflect the extent of oxidative stress (Bartsch and Nair, 2000) Antibodies, both poly- and mono-clonal, have been generated against RCS-modified DNA bases. For example, MDA-deoxyguanosine (Sevilla *et al.*, 1997), deoxycytidine-glyoxal (Mistry *et al.*, 2003). These specific RCS-DNA antibodies have been successfully used to measure carbonyl stress in human and animal studies following oxidative and peroxidative insult (Cooke *et al.*, 2003).

Carbonyls produced via lipid peroxidation

The peroxidation of membrane-derived lipid molecules is a well-studied consequence of increased intracellular oxidant levels (Esterbauer *et al.*, 1991). The most

commonly characterized products are aldehydes, derived from ω- 6 polyunsaturated fatty acids, such as malondialdehyde (MDA), hexanal, acrolein, glyoxal, crotonaldehyde, trans-2-nonenal, 4-oxo-2-nonenal and 4-hydroxy-2- nonenal (HNE) (Esterbauer *et al.*, 1991). MDA is the most common aldehyde produced, comprising of 70% of the total produced by lipid peroxidation (Esterbauer *et al.*, 1991). Hexanal contributes 15% and HNE contributes 5% of total aldehydes (Benedetti *et al.*, 1980). Acrolein was identified as a lipid peroxidation product more recently through studies that examined the oxidation of low density lipoprotein (LDL) but was previously characterized as an environmental pollutant (Uchida *et al.*, 1998).

Carbonyls produced via glycoxidation

Reducing sugars such as glucose can form Schiff bases with amino groups on the amino acids lysine and arginine, through the Maillard reaction. This can, through a series of rearrangements, give rise to advanced glycation endproducts (AGEs) (Munch *et al.*, 1998). Oxidation of these glycation products can release dicarbonyls, such as the α-oxoaldehydes, MG, glyoxal, and 3-deoxyglucosone, as well as short-chain aldehydes, such as diacetyl, acetol, pyruvaldehyde and acrolein (Thornalley, 2005).

Reactivity of carbonyls

Many of the carbonyls that are produced as a result of either lipid peroxidation or glycoxidation are extremely reactive alkanals, such as hexanal, are the least reactive and have weaker effects than unsaturated aldehydes. Alkenals containing a C = C unsaturated bond, such as acrolein, are usually an order of magnitude more reactive than the alkanals. This is particularly the case if they contain an α,β- unsaturated (C2–C3) double bond, in addition to the C1 aldehyde. This makes the C3 carbon a strong electrophile that undergoes Michael addition by nucleophilic groups on proteins, DNA and lipids (Marnett *et al.*, 1985), thereby causing damage to these molecules. The aldehyde group is also reactive and can form Schiff bases with amino acids. 4-hydroxy-2-alkenals, such as HNE, are extremely reactive because of the interaction between the electrophilic double bond, the aldehyde moiety and the hydroxyl group (Witz, 1989).

Cytotoxicity of reactive carbonyls

Cytotoxicity is generally measured by examining loss of viability. Reactive aldehydes, such as HNE, interact directly with proteins and membranes, causing

significant loss of function to membrane transporters, enzymes, signalling components, transcription factors, microtubules and other proteins, such as tau (Karlhuber *et al.*, 1997, Picklo *et al.*, 2002). Acrolein is also cytotoxic and, in neuronal cells, causes changes in Ca^{2+} concentrations, altering glucose transport and glutamate uptake (Li *et al.*, 1997, Lovell *et al.*, 2001). As described earlier, other aldehydes, such as MG, can rapidly form Schiff bases with amino acids, which leads to the production of AGE at a much faster rate than from sugars, such as glucose (Thornalley, 1996). MG therefore causes significant toxicity to a range of cell types, including neuronal cells (Suzuki *et al.*, 1998).

Antibody production against RCS

DNA adducts formed by RCS are known to possess strong miscoding potential *in vitro* and of the DNA adducts formed by MDA, pyrimido (1,2α) purin-10(3H) one (M_1G) is readily detected in many human tissues. Other propano-adducts also originate from α, β-unsaturated aldehydes or enals such as acrolein (Acr), crotonaldehyde (Cro), glyoxal (Gly) and 4- hydroxyl-nonenal (HNE). Adducts formed by HNE have been detected in healthy human colon tissue (Wacker *et al.*, 2000). Etheno-DNA adducts are formed from the epoxides of enals and elevated levels of this type reflect the extent of oxidative stress (Bartsch and Nair., 2000). Antibodies, both poly- and monoclonal, have been generated against RCS-modified DNA bases. For e.g., MDA-deoxyguanosine (Sevilla *et al.*, 1997), deoxycytidine-glyoxal (Mistry *et al.*, 2003) deoxyadenosine-acrolein (Kawai *et al.*, 2003), 1, N (6)-ethenodeoxyadenosine (Frank *et al.*, 2004) and ethenodeoxyguanosine (Foiles *et al.*, 1993). These specific RCS-DNA antibodies have been successfully used to measure carbonyl stress in human and animal studies following oxidative and peroxidative insult (Zhang *et al.*, 2002, Cooke *et al.*, 2003).

Methylglyoxal

Methylglyoxal (MG) is a metabolite of sugar and is highly reactive α-oxoaldehyde. It is a small molecule with molecular weight of 72. MG has a ketone group and an aldehyde moiety. The aldehyde group is more reactive than the ketone. It is a yellow liquid with characteristic pungent odor. It has 3 forms in aqueous

solution: unhydrated (1%), monohydrate (71%) and dehydrate (28%), which are in rapid equilibrium (Rae, *et al.*, 1990).

Methylglyoxal is inevitably produced in the course of metabolism even under normal conditions. It is formed mainly from the spontaneous transformation of triose phosphates. Therefore, MG is an intrinsic component of the glycolytic pathway in mammalian cells, including vascular smooth muscle cells (VSMCs) (Ekblom, 1998). An increased MG formation may occur because of an increased availability of precursors such as increased plasma glucose or administration of ethanol or threonine (Thornalley and Tisdale, 1988). It is also formed during the non-enzymatic glycation, an early stage of the Maillard reaction (Monnier and Cerami, 1981), which is one of the post-translation modification processes between free reducing sugars and free amino groups of proteins. This compound is more reactive than parent sugar with respect to its ability to react with amino groups of proteins to form cross-links and AGEs (Brownlee *et al.*, 1988). MG has been implicated in secondary diabetic complications promoting formation of AGEs (Uchida *et al.*, 1998). On the other hand, MG is detoxified by the ubiquitous glyoxalase system that highly relies on the cellular level of reduced glutathione (GSH). Reduced availability of GSH also contributes to the increased levels of MG. Numerous studies showed that levels of MG were elevated in patients with diabetes mellitus (Beisswenger *et al.*, 1999). MG levels were correlated with the glycated hemoglobin (HbA1c) (Thornalley *et al.*, 1989) and reflected glycemic fluctuation (Nemet *et al.*, 2005) in diabetic patients.

Endogenous MG formation

Major Pathway

MG is produced during the metabolism of carbohydrates, lipids and proteins. Several enzymatic or non-enzymatic pathways are involved in the endogenous formation of MG (Fig.1). Endogenous MG is formed from metabolic intermediates of carbohydrates, proteins and fatty acids. The majority of MG is derived from the metabolites of carbohydrate, such as glucose and fructose. MG is formed during glycolysis. Glucose is phosphorylated by glucokinase to form glucose-6-phosphate (G-6-P). This reaction decreases the intracellular glucose levels and promotes continuous transportation of glucose into the cell through the glucose transporter on

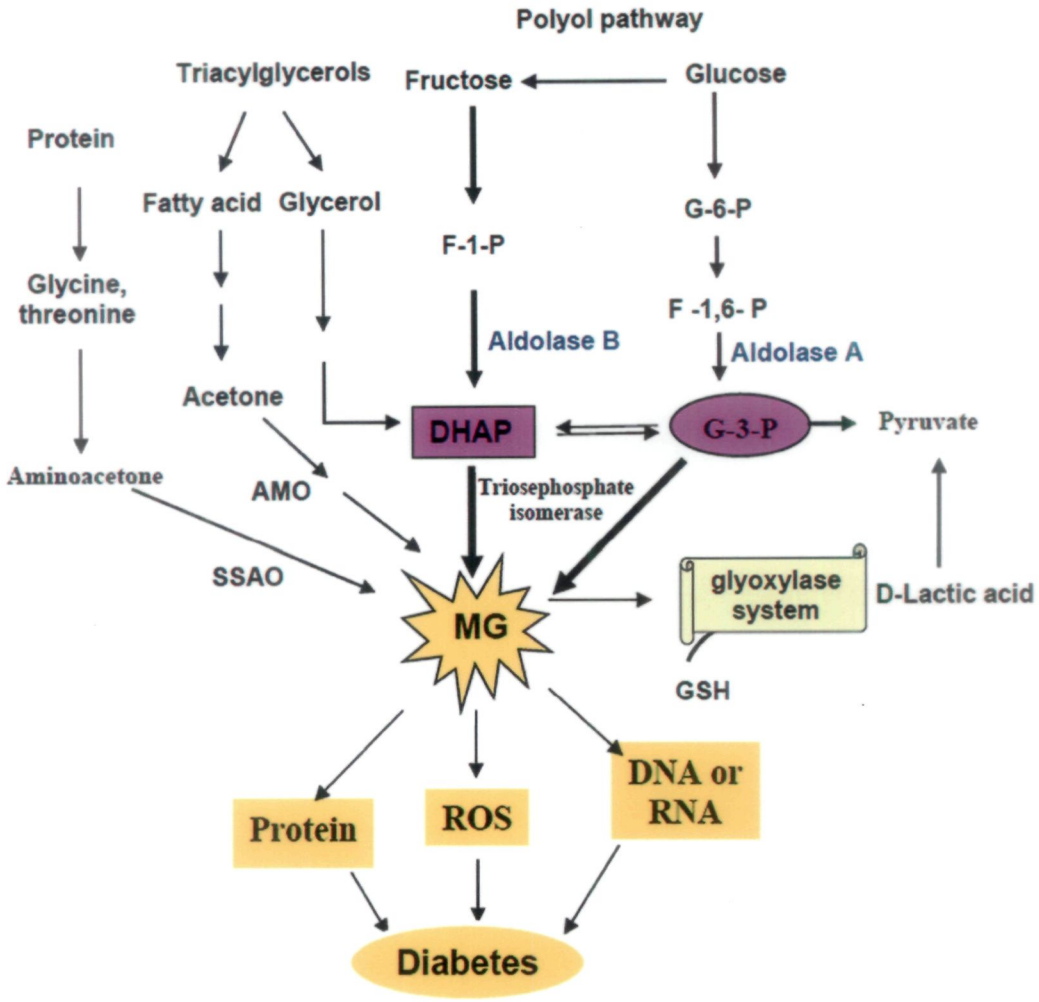


Fig. 1 **Formation, metabolism and toxicity of methyglyoxal.**

Source: Adapted from Wang, H., Thesis, University of Saskatchewan, 2009.

AMO : Acetol monooxygenase
GSH : Reduced glutathione
MG : Methylglyoxal
SSAO : Semicarbazide-sensitive amine oxidase

the cell membrane. G-6-P is then converted to fructose-6-phosphate (F-6-P) via glucose phosphate isomerase. This step is reversible but easily driven to F-6-P due to the lower levels of F-6-P. Subsequently, fructose-1, 6- biphosphate (F-1,6-P) is irreversibly formed from F-6-P and G-6-P due to catalysis by phosphofructokinase-1 (PFK-1). This reaction is the key point in the glycolytic process. F-1,6-P, then, is split by aldolase into two triose sugars, dihydroxyacetone phosphate (DHAP), a ketone, and glyceraldehyde-3-phosphate (G-3-P). DHAP and G-3-P can spontaneously convert to MG (Phillips and Thornalley, 1993).

MG is mainly formed nonenzymatically from DHAP and G-3-P, and the non-enzymatic formation of MG occurs in all cells and organisms. For example, MG formation in human red blood cells *in vitro* under normal glycemic conditions is due to nonenzymatic fragmentation of triosephosphates (Phillips and Thornalley, 1993).

The formation of MG in early glycation was investigated by Thornalley *et al.* (Thornalley *et al.*, 1999). Glucose (50 mM) degraded slowly at 37 °C to form MG throughout a period of 3 weeks. Therefore, a short period of hyperglycemia may be sufficient to induce MG formation *in vivo* (Thornalley *et al.*, 1999). Bovine retinal endothelial cells exposed to D-glucose (30 mM) for 7 days produced significantly higher levels of MG than cells cultured with L-glucose or control cells (Padayatti *et al.*, 2001). In addition, high glucose caused increased MG formation and MG modification of the corepressor mSin3A in mouse kidney endothelial cells. Consequently, MG-modified mSin3A mediated high glucose-induced expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in cells and high sensitivity of endothelial cells to tumour necrosis factor-α (TNF-α). It was shown that glucose induced vascular inflammation and disease via the formation of MG (Yao *et al.*, 2006).

Fructose as a precursor for MG has been investigated *in vivo* (Wang *et al.*, 2008). Increased serum and aortic levels of MG have been observed in fructose-fed rats (Wang *et al.*, 2008). MG induced structural remodeling in mesenteric artery and ROS production in aorta of fructose-fed rats. MG is also responsible for the high blood pressure and hypertriglyceridemia seen in those rats. In addition, MG impairs insulin signaling in adipose tissue of fructose-fed rats through decreasing insulin-induced insulin-receptor substrate-1 (IRS) tyrosine phosphorylation and reducing the

activity of phosphatidylinositol (PI) 3-kinase (Jia and Wu, 2007). MG can also be formed enzymatically from G-3-P and DHAP. Triosephosphate isomerase hydrolyses G-3-P and DHAP and removes phosphate to yield MG (Pompliano *et al.*, 1990).

Minor pathways

MG is a byproduct of acetone metabolism. Acetone monooxygenase catalyzes acetone to acetol, and acetol monooxygenase (AMO) converts acetol to MG (Casazza *et al.*, 1984). In addition, formation of MG is also found during the metabolism of aminoacetone, which is a metabolite of proteins. Semicarbazide-sensitive amine oxidase (SSAO) is able to convert aminoacetone into MG (Lyles, 1996). Increased serum SSAO activities have been found in patients with diabetes and vascular disorders, and treatment with selective SSAO inhibitors reduced atherogenesis in diabetic mice fed with a high-cholesterol diet (Yu *et al.*, 2003).

MG in food and beverages

MG is formed in food and beverages during the processing, cooking and prolonged storage (Nemet *et al.*, 2006). MG can be formed from carbohydrates by fragmentation of the sugar moiety during retro-aldol condensation and auto-oxidation. In addition, the formation of MG was observed during the heating process of glucose, fructose and maltose, where the amount of MG obtained from monosaccharides was markedly higher than that from disaccharides (Nemet *et al.*, 2006). Moreover, decomposition of different lipids, caused by storage and processing, can also affect the accumulation of MG in food. A broad range of MG levels was obtained during accelerated storage (60°C for 3 and 7 days) or cooking (200°C for 1 h) of oil, depending on oil origin (salmon, cod liver, soybean, olive and corn oils) (Fujioka and Shibamoto, 2006). For instance, the formation of MG ranged from 2.03 ppm in cod liver oil to 2.89 ppm in tuna oil heated at 60 °C for 7 days. However, olive oil is the only vegetable oil that yields MG under the accelerated storage conditions.

Coffee is a widely consumed beverage. It is interesting to know whether MG is present in green and roasted coffee beans. The amount of MG is small in green coffee beans, but increases in the early phases of the roasting process and then declines. Thus, mild or medium toasted coffee beans have the highest amount of MG

content (Daglia *et al.*, 2007). About 100 μg of MG has been determined to be present in one gram of coffee. MG owns the strongest mutagenicity in dicarboyls in coffee (Nagao *et al.*, 1986). Besides food and beverages, drinking water can also be an exogenous source of MG. Ozonation and chlorination of natural water, the applied process in the treatment of drinking water, can lead to the formation of MG (Matsuda *et al.*, 1992).

Toxicity of MG

MG is inextricably linked to oxidative stress

Generation of reactive oxygen species (ROS) is associated with MG metabolism. Production of MG from acetone and aminoacetone yields hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot-}$) as by products, and degradation of MG by glyoxal oxidase or photolysis produces H_2O_2 and other radical species (Kalapos, 2008). In addition, treatment of various cell types with MG induces their ROS production (Kalapos, 2008). MG also hinders antioxidant defence by reacting with functional thiol groups of glutathione (GSH) and plasma albumin to reduce their activity (Faure *et al.*, 2005; Kalapos, 2008). MG inhibits the antioxidant enzyme superoxide dismutase by altering its structure (Kang, 2003) and impedes glyoxalase and GSH peroxidase function. Through above mechanisms, MG promotes oxidative stress in cellular systems and encourages its own survival. Oxidative stress is implicated in apoptosis of pancreatic β-cells and impaired insulin signalling in diabetes (Newsholme *et al.*, 2007), as well as in cell and tissue damage. Whereas limited amounts of ROS are used by the immune system for functions, such as signalling and microbicidal activity, unregulated ROS production leads to oxidative protein and DNA damage, which promotes tissue dysfunction, apoptosis and premature cell aging. Cell aging can encourage ill health, as senescence of immune cells is associated with increased incidence of infection and cancer (Martin and Grotewiel, 2006).

Modification of protein

Under physiological conditions, more than 90% of MG is bound reversibly with cellular proteins (Lo *et al.*, 1994). MG reacts with arginine, lysine and cysteine

residues of proteins to form advanced glycation endproducts (AGEs). Arginine-derived hydroimidazolone and lysine-derived N-ε-carboxyethyl-lysine (CEL) and N-ε-carboxymethyl-lysine (CML) are products of irreversible reactions of protein residues with MG (Lo *et al.*, 1994). The concentration of AGEs in mammalian tissues, plasma and extracellular matrix *in vivo* depends on the protein substrate, tissue location and type of AGEs. For instance, the highest concentration of hydroimidazolone was found in the lens of older individuals and CML accumulates on lens, skin and cartilage (Ahmed *et al.*, 1997; Verzijl *et al.*, 2000).

MG-induced AGEs are involved in the pathogenesis of many diseases, such as diabetes, hypertension and neurodegenerative diseases (Desai and Wu, 2007; Munch *et al.*, 2003). AGEs induce cross-linkage of proteins to decrease arterial and myocardial compliance and promote vascular stiffness, leading to the alteration of vascular structure and function, which contributes to the development of hypertension and diabetic vascular complications (Goh and Cooper, 2008). AGEs also have been seen accumulated in diabetic kidney, retina and atherosclerotic plaques (Makita *et al.*, 1994; Bucala and Vlassara, 1995; Hammes *et al.*, 1999), and are closely linked to the development of diabetic complications. In addition, AGEs interact with some receptors, like the receptor for AGEs (RAGE), where they interfere with cell signaling and nuclear factor-κB (NF-κB) mediated pathway, leading to enhanced oxidative stress and generation of proinflammatory cytokines.

MG-induced AGEs formation impairs anti-oxidant enzymes, leading to the excessive accumulation of reactive oxygen species (ROS). Arginine, lysine and cysteine are residues involved in the active sites of enzymes, and the irreversible reaction of MG with residues may alter the activity of those enzymes. For example, activities of glutathione reductase and glutathione peroxidase were reduced significantly, accompanied by the increased MG-induced AGEs formation in aorta from adult SHR (Wang *et al.*, 2005). MG also modifies Cu/Zn-SOD by covalent cross-linking of the proteins, leading to the release of copper ions from the enzyme and the inactivity of the enzyme (Kang, 2003). Furthermore, decreased extracellular SOD activity was due to excessive glycation, not to the impaired synthesis of this enzyme in patients with diabetes (Ciechanowski *et al.*, 2005). Aminoguanidine, a scavenger of MG and AGEs, increased the activities of catalase, glutathione reductase

and glutathione peroxidase in insulin-dependent diabetic rats and prevented the impairment of blood antioxidant systems (Stoppa *et al.*, 2006).

Modification of nucleic acid

MG can be a mutagen since it modifies nucleotides poly A, poly G and poly C, but not poly-U (Krymkiewicz, 1973). MG inhibited skin cell proliferation and caused extensive DNA strand cleavage by the extensive formation of DNA-protein cross-links (Roberts *et al.*, 2003). MG-induced cytotoxicity and mutation were concentration dependent. Multi-base deletions were predominant (50%) in MG-induced mutations, followed by base-pair substitutions (35%), in which G:C to C:G and G:C to T:A transversions were predominant (Murata-Kamiya *et al.*, 2000). Furthermore, MG increased point mutations in *Salmonella typhimurium* (Migliore *et al.*, 1990), and the occurrence of point mutations correlated with the glycation rate of DNA (Pischetsrieder *et al.*, 1999). The cross-link formation of protein with DNA by glycation with MG has been investigated. A protein-DNA cross-link was observed after 90 min exposure to MG (1.5 mM) in Chinese hamster ovary cells (Brambilla *et al.*, 1985). In addition, MG cross-linked a guanine residue of the substrate DNA and lysine and cysteine residues near the binding site of the DNA polymerase during DNA synthesis, and that DNA replication was severely inhibited by the MG-induced DNA-DNA polymerase cross-link in *E.coli* (Murata-Kamiya and Kamiya, 2001).

Apart from these modifcations, DNA-AGEs are also formed by the reaction of MG and nucleic acids. The major AGE adducts found in DNA are MG-derived dG modifications (Bidmon *et al.*, 2007 and Synold *et al.*, 2008), leading to the formation of carboxyethyl deoxyguanosine (CEdG). CEdG is the major adduct of the glycation reaction of MG and DNA. MG-glycation of dG residues seems to increase the frequency of dG deletion (Seidel and Pischetsrieder, 1998), leading to abasic sites, which are potentially mutagenic. MG treatment of cells leads to reduced DNA replication and increased mutations, the most prominent being multibase deletions and base-pair substitutions (Murata-Kamiya *et al.*, 2000). MG also has the potential to crosslink DNA to proteins, for example between the dG of DNA and Lys or Cys residues close to the DNA polymerase binding site (Murata-Kamiya and Kamiya, 2001). Therefore, any mutations or DNA damage/cross-linking caused by MG could

result in reduced or altered transcription, which might affect genes/proteins involved in immune defence or any other bodily function. Furthermore, because PARP plays a role in DNA repair and is activated by DNA damage, dG glycation or other MG-related DNA damage could also negatively impact glycolysis.

Scavengers of MG

To date, specific MG scavengers are not available in the market, but some agents like aminoguanidine, alagebrium and metformin including some AGE inhibitors like D-lysine, D- penicillamine, diclofenac and desferrioxamine are capable of reducing MG levels. In addition to these scavengers, vitamins like pyridoxamine, thiamine and thiamine pyrophosphates, pyridoxal phosphate also play crucial role in inhibiting post amadori glycation cascade and as carbonyl trapping AGE inhibitors. These inhibitors are currently used in different studies, although the mechanism is unclear.

Physiological and pathological levels of MG

The levels of MG in plasma of normal human subjects vary from 123 nM to 650 nM depending on different studies (Beisswenger, *et al.*, 1999; Odani, *et al.*, 1999; Lapolla, *et al.*, 2005; Nemet, *et al.*, 2005). The inconsistency of those values seems dependent on different methods used to test the compound.

Elevated levels of MG have been observed in different kinds of diseases, such as hypertension, diabetes and renal failure. MG was significantly elevated in patients with diabetes mellitus versus normal subjects (212 nM vs. 160 nM) (Beisswenger, *et al.*, 1999). The data from another laboratory indicated that plasma MG levels were significantly higher in patients with diabetes mellitus (194 ng/ml) and patients with chronic renal failure (128 ng/ml) than those from normal subjects (59 ng/ml) (Odani *et al.*, 1999). In addition, MG levels were significantly elevated in patients with end-stage renal disease versus normal controls (24 vs. 9 μg/ml) (Lapolla, *et al.*, 2005).

Numerous studies demonstrated that MG played an important role in the pathogenesis of diabetes and diabetic complications. It has been observed that the plasma levels of MG in diabetic patients were significantly increased compared with those in

normal controls (742 ± 141 vs. 520 ± 42 nM,). MG was also a parameter reflecting glycemic fluctuation (Nemet, *et al.*, 2005).

Diabetes mellitus

Advances in clinical science over a single professional lifetime during the second half of the 20th century have led to improvements in understanding the causes and complications of diabetes, together with alleviation of suffering to an extraordinary degree. Diabetes mellitus refers to a group of common metabolic disorders that share the phenotype of hyperglycemia. Several types of diabetes mellitus exist and are caused by a complex interaction of genetics and environmental factors, viral infection and autoimmune disease have been implicated (Paik *et al.*, 1982; Kataoka *et al.*, 1983; Sandler *et al.*, 2000; Shewade *et al.*, 2001). While exogenous insulin and other medications can control many aspects of diabetes, numerous complications affecting the vascular system, kidney, retina, lens, peripheral nerves and skin are common and are extremely costly in terms of longevity and quality of life. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications (Baynes *et al.*, 1999). Diabetes is usually accompanied by increased production of free radicals (Chang *et al.*, 1993; Young *et al.*, 1995; Baynes *et al.*, 1999) or impaired antioxidant defenses (Halliwell and Gutteridge, 1990; McLennan *et al.*, 1991; Saxena *et al.*, 1993). Mechanisms by which increased oxidative stress is involved in the diabetic complications are partly known, including activation of transcription factors, advanced glycated end products (AGEs) and protein kinase C. Depending on the etiology of the diabetes mellitus, factors contributing to hyperglycemia include reduced insulin secretion, decreased glucose utilization and increased glucose production. The metabolic dysregulation associated with diabetes mellitus causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the individual with diabetes. In the United States, diabetes mellitus is the leading cause of end-stage renal disease (ESRD), non traumatic lower extremity amputations, and adult blindness (Powers, 2008). It also predisposes to cardiovascular diseases. In diabetes mellitus, low insulin levels prevent cells from absorbing glucose, as a result glucose builds up in the blood. When glucose-laden blood passes through kidneys, all the excess glucose cannot be absorbed. This excess

glucose secreted in urine along with water and electrolytes as well as ions required by cells to regulate the electric charge and flow of water molecules across the cell membrane. This causes polyurea, polydipsia and weight loss as classical symptoms of the diabetes. These symptoms together with a random plasma glucose concentration ≥11.1 mmol/L (200mg/dL) is sufficient for the diagnosis of diabetes mellitus although fasting plasma glucose is the most reliable and convenient test for identifying diabetes in asymptomatic individuals (Powers, 2008).

Classification of diabetes mellitus

Diabetes mellitus is classified on the basis of the pathogenic process that leads to hyperglycemia (American Diabetes Association, 2007). The two broad categories of diabetes mellitus are designated as type I and type II. Both types of diabetes are preceded by a phase of abnormal glucose homeostasis as the pathogenic processes progresses. Type I diabetes is the result of complete or near-total insulin deficiency. Type II is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production. Distinct genetic and metabolic defects in insulin secretion give rise to the common phenotype of hyperglycemia in type II diabetes mellitus and have important potential therapeutic implications now that pharmacologic agents are available to target specific metabolic derangements. Type II diabetes mellitus is preceded by a period of abnormal glucose homeostasis classified as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). The term insulin-dependent diabetes mellitus (IDDM) and noninsulin-dependent diabetes mellitus (NIDDM) are obsolete. Since many individuals with type II diabetes eventually require insulin treatment for control of glycemia, the use of term NIDDM generated considerable confusion. Other etiologies for diabetes mellitus include specific genetic defects in insulin secretion or action, metabolic abnormalities that impair insulin secretion, mitochondrial abnormalities and a host of conditions that impair glucose tolerance. The etiologic classification of diabetes mellitus is illustrated in Table 1. Maturity onset diabetes of the young (MODY) is a subtype of diabetes mellitus characterized by autosomal dominant inheritance, early onset of hyperglycemia (usually < 25 years), and impairment in insulin secretion (Powers, 2008).

TABLE 1

Etiologic classification of diabetes mellitus

I. **Type I diabetes** (β-cell destruction, usually leading to absolute insulin deficiency)

A. Immune Mediated.
B. Idiopathic.

II. **Type II diabetes** (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly insulin secretory defect with insulin resistance).

III. **Other specific types of diabetes**

A. *Genetic defects of β- cell function characterized by mutations in:*

1. Hepatocyte nuclear transcription factor (HNF) 4α (MODY 1).
2. Glucokinase (MODY 2).
3. HNF-1 α (MODY 3).
4. Insulin promoter factor-1 (IPF-1; MODY 4).
5. HNF-1 β (MODY 5).
6. Neuro D1 (MODY 6).
7. Mitochondrial DNA

B. *Genetic defects in insulin action*

1. Type A insulin resistance
2. Leprechaunism
3. Rabson- Mendenhall syndrome

C. *Diseases of the exocrine pancreas*

1. Pancreatitis
2. Pancreatectomy
3. Neoplasia
4. Cystic Fibrosis
5. Hemochromatosis
6. Fibrocalculous pancreatopathy

D. *Endocrinopathies*

1. Acromegaly
2. Cushing's syndrome
3. Glucagonoma
4. Pheochromocytoma
5. Hyperthyroidism
6. Somatostatinoma

E. *Drug- or chemical-induced*

1. Pentamidine
2. Nicotinic acid
3. Glucocorticoids
4. Thyroid hormone
5. Diazoxide
6. β- adrenergic agonists
7. Phenytoin
8. α- interferon
9. Protease inhibitor

F. *Infections*

1. Congenital rubella
2. Cytomegalovirus
3. Coxsackie

G. *Uncommon forms of immune-mediated diabetes*

1. "Stiff Person" syndrome
2. Anti-insulin receptor antibodies

H. *Other genetic syndromes sometimes associated with diabetes*

1. Down's syndrome
2. Klinfelter's syndrome
3. Turner's syndrome
4. huntington's chorea
5. Myotonic dystrophy
6. Porphyria

IV. **Gestational Diabetes Mellitus (GDM)**

Source: Adapted from American Diabetes Association, 2007.

Type I diabetes mellitus

Absolute insulin deficiency caused by autoimmune-mediated destruction of pancreatic β-cells characterizes type I diabetes. This condition is also called "insulin-dependent diabetes" or "juvenile diabetes." The main cause of the beta cell loss is a T-cell mediated immune attack (Rother, 2007). It is thought to be caused by a combination of environmental factors and viral infection, superimposed on a genetic susceptibility. It accounts for ~10% of those with diabetes in the United States, but the prevalence may be increasing. The disorder may be further sub classified into type IA if autoimmune markers are found, usually at the time of diagnosis (A Report on Diabetes care, 1997) Type IB diabetes is an absolute insulin deficiency in which no autoimmune markers can be identified. Type IB diabetes may be more common in people of Asian heritage (Abiru *et al.*, 2002). Type I diabetes is a multifactoral autoimmune disease thought to arise from a complex interaction between both genetic susceptibility and environmental insult(s). Several autoantibody (Table 2) markers have been detected in autoimmune diabetes including islet cell antibodies (ICA), insulin autoantibodies (IAA) (Atkinson *et al.*, 1992), glutamic acid decarboxylase-65 (GAD-65) autoantibodies (Jun *et al.*, 2002) and antibodies to tyrosine phosphatases IA-2 and IA-2β (Lan *et al.*, 1996; Lu *et al.*, 1996). There is a strong association of type I diabetes with individuals who possess particular HLA haplotypes. HLA DR4-DQ8 and DR3-DQ2 are present in > 90% of children with type IA diabetes (Powers, 2008). Furthermore, 30–50% of patients with type IA diabetes are heterozygotes for HLA DR4-DQ8 and DR3-DQ2, whereas this combination of alleles is only present in ~2.4% of the general population (Devendra and Eisenbarth, 2003). Most people with these HLA alleles do not develop type I diabetes, demonstrating that other factors are involved in the development of the disease. Many triggers have been proposed for the development of type I diabetes in genetically susceptible individuals. Viruses such as enteroviruses, coxackie virus and rubella have been proposed as culprits but have not been definitively shown to induce type I diabetes (Lammi *et al.*, 2005). Food additives or toxins, such as nitrosamines, have also been proposed as a cause of diabetes (Helgason *et al.*, 1981). Some investigators have also implicated cow's milk as an initiating factor in the development of autoimmunity in type I diabetes (Oute *et al.*, 1999). Whatever initiating mechanism is, the autoimmune destruction of β-cells leads

TABLE 2

Major autoantigens in type I diabetes

S. No.	Characteristics	GAD65	IA-2	Insulin
1	Amino Acid length	585	979	51
2	Molecular weight (Da)	65,000	106,000	6,000
3	Chromosome	10p11	2q35	11p15
4	Cell type in which expressed	Neuroendocrine pancreatic islet cells	Neuroendocrine pancreatic islet cells	Pancreatic islet β cell
5	Intracellular location	Neuron-like small vesicles	Secretory vesicles	Secretory vesicles
6	Function	Converts glutamic acid to GABA; inhibitory neurotransmitter	Enzymatically inactive member of PTP family	Ligand for the insulin receptor; regulation of blood glucose

Source: Notkins & lernmark, J. Clin. Invest, **108**, 2001.

to a progressive decline in the body's insulin secretory capacity. Eventually, this decline manifests itself in hyperglycemia after a large carbohydrate load, such as a meal or a glucose tolerance test. When ~80% of β-cells have been destroyed, patients develop the first clinical symptoms of diabetes. Interestingly, the rate of β-cell decline can vary based on age, with older patients who develop type I diabetes typically experiencing a much more gradual decline in β-cell mass (Powers, 2008).

Type II diabetes mellitus

Type II diabetes is a heterogeneous group of conditions that constitute ~90% of diabetes. Like type I diabetes, type II diabetes also involves both genetic susceptibility and environmental factors, although the genetic component may be greater than in type I diabetes. It is caused by a combination of insulin resistance and relative insulin deficiency with increased hepatic glucose production. It is important to note that some individuals experience predominantly insulin resistance and others insulin deficiency. Insulin resistance is generally thought to precede insulin deficiency. Obesity is associated with increased insulin resistance and may be the reason that, type II diabetes is more common in obese individuals. The precise mechanism by which obesity leads to insulin resistance is not completely described but may be related to several biochemical factors, such as free fatty acids, leptin, tumour necrosis factor-α and other substances. In addition, many genetic polymorphisms may play a part in insulin resistance, possibly through post-insulin receptor signal transduction mechanisms (Powers, 2008). Overweight and obesity are strongly associated with development of type II diabetes and may be responsible for the majority of the growing diabetes pandemic (Wannamethee and Shaper 1999). Furthermore, weight loss is strongly associated in prospective studies with decreased progression from impaired glucose tolerance (IGT) to type II diabetes (Knowler *et al.*, 2002). Insulin resistance alone, however, does not cause diabetes. Most obese people do not develop type 2 diabetes, despite increased insulin resistance (Polansky, 2000). For type II diabetes to emerge, there must also be relative insulin deficiency. Before type II diabetes develops, the pancreatic β-cells increase their production of insulin to compensate for increased insulin resistance. It has been proposed, that there is measurable β-cell hypertrophy present in obese subjects who do not have diabetes. For unclear reasons, β-cell secretory capacity gradually declines in some individuals,

leading to the development of type II diabetes. As β-cell insulin secretory capacity declines, type II diabetes begins to develop. Initially, hyperglycemia is only observed after large meals, as in type II diabetes. As β-cell function declines further, however, hyperglycemia becomes more severe. Studies have suggested that 40% of β-cell mass may be lost in individuals who have glucose intolerance, and ~60% may be lost when clinical type II diabetes develops (Butler *et al.*, 2003). Hepatic insulin resistance and relative insulin deficiency also lead to increased hepatic gluconeogenesis, which further worsens hyperglycemia. Eventually, the degree of hyperglycemia worsens and becomes virtually universal if left untreated (Powers, 2008). The cause of β-cell failure in type II diabetes is unknown. In addition to a genetic predisposition, studies have also demonstrated higher rates of apoptosis and decreased β-cell mass in patients with type II diabetes (Butler *et al.*, 2003). There are also increased amounts of amyloid deposits in the islets of patients with type II diabetes (Khan *et al.*, 1999). Several authors speculate that increased insulin resistance may be a genetic trait that can be worsened by obesity and that β-cells compensate for this increased resistance. Some individuals, however, cannot maintain this compensation because of β-cell failure, which leads to the development of type II diabetes.

Asian Indians are more prone to type II diabetes and premature coronary artery disease due to "Asian Indian Phenotype". The phenotype refers to certain unique clinical and biochemical abnormalities in Indians which include increased insulin resistance, greater abdominal adiposity i.e., higher waist circumference despite lower body mass index, lower adiponectin and higher high sensitive C-reactive protein levels (Mohan *et al.*, 2007).

Methylglyoxal and diabetes mellitus

No matter the cause of diabetes, the result is always hyperglycaemia. This excess glucose metabolism drives several damage pathways and raises concentrations of the reactive dicarbonyl, methylglyoxal (MG). MG can modify the structure and function of target molecules by forming advanced glycation end-products (AGEs) that act through their receptor (RAGE) to perpetuate vascular and neuronal injury responsible for long-term complications of diabetes. Diabetes patients suffer lower resistance to many common infections, although the cause for this lower resistance

remains elusive. It has been suggested that MG could be a missing link between hyperglycaemia and immune suppression in diabetes. The glycolytic by-product methylglyoxal (MG) is a potent modifier of immune components and function, inducing immune damage which might provide a link between hyperglycaemia and diabetes-related infection risk (Claire and Stella, 2009).

MG and metabolic dysfunction in diabetes

The reactive α-oxoaldehyde MG is formed as a natural by-product of several metabolic pathways, mainly from glycolysis but also from lipid peroxidation and threonine catabolism (Kalapos, 1999). To prevent cellular damage, MG is detoxified by defence components, including the specialised glyoxalase system, which converts α-oxoaldehydes to their respective hydroxyacids (Thornalley, 2003). In diabetes, the increased flux of glucose metabolism causes metabolic dysfunction. Higher rates of respiration through the electron transport chain within mitochondria lead to superoxide leakage, increased oxidative stress and activation of the nuclear enzyme, poly (ADP-ribose) polymerase-1 (PARP). PARP activation depletes its substrate NAD^+ (slowing rate of glycolysis and electron transport) and inhibits glyceraldehyde-3-phosphate (G-3-P) dehydrogenase (GAPDH) (Pacher and Szabo, 2005). By inhibiting the GAPDH conversion of G-3-P, glycolytic intermediates build up, compounded by increased glucose at the head of the chain. Glycolytic intermediates are pushed down their respective metabolic pathways (protein kinase C, polyol and hexosamine), altering cellular balance and causing damage through raised angiogenic factors, reduced nitric oxide and altered gene expression and protein function (Rolo and Palmeira, 2006). This damage, along with that from formation of advanced glycation end-products (AGEs), lies behind the vascular and neuronal complications of diabetes. The glycolytic intermediates fructose-1,6-diphosphate, G-3-P and glycerol phosphate are direct precursors of MG; thus, in diabetes, MG production is vastly increased. Furthermore, the glyoxalase defence against MG becomes overwhelmed, with activity of glyoxalase I decreased by oxidative stress and MG concentrations are able to rise (Thornalley, 2008). Therefore, MG blood levels are 2–6 times higher in diabetes patients compared with controls (McLellan *et al.*, 1994). Because MG initially binds reversibly to tissues *in vivo* (Lo *et al.*, 1994), actual levels might be far higher (up to 100 times higher) than this measurement suggests and

concentrations probably vary locally depending upon availability of precursors and activity of defences.

Role of MG in diabetes mellitus

MG-induced AGEs in diabetes mellitus

MG is the most important precursor of AGEs. Numerous studies show that accumulation of intracellular MG and formation of AGEs alter cell function and contribute to the development Of type II diabetes mellitus and diabetic complications such as atherosclerosis, nephropathy, and retinopathy.

In cultured endothelial cells, MG accumulated rapidly under hyperglycemic conditions (Shinohara, *et al.*, 1998). In addition, serum levels of AGEs increased in patients with type II diabetes and coronary artery disease (Kilhovd, *et al.*, 1999). AGEs induce diabetic atherosclerosis by multiple ways. Argpyrimidine, the fluorescence product of the reaction of MG with arginine residues in protein, has been demonstrated to localize in atherosclerotic lesions, fatty streaks, lipid containing SMCs and macrophages in diabetic patients (Friedman, 1999; Oya, *et al.*, 1999). A correlation of AGEs and severity of atherosclerotic lesions was also shown. AGEs decrease NO availability by quenching NO, impair LDL removal by trapping LDL in the sub endothelium and decrease LDL 39 receptor recognizing AGEs-modified LDL (Bucala, *et al.*, 1994). Furthermore, AGEs enhanced VCAM-1 expression by activating NF-κB. VCAM-1 stimulates the migration of monocytes through endothelium, which is the first step of atherogenesis (Kunt, *et al.*, 1999).

The kidney is a key target of MG and AGEs mediated damage. Mouse renal damage was found after oral administration of MG. A 5-month treatment with MG resulted in elevated levels of collagen in kidney and increased glomerular basement membrane thickness (Golej, *et al.*, 1998). Diabetic mice have significantly elevated renal AGEs, and these abnormalities have been linked to various structural aspects of diabetic nephropathy, including glomerular basement membrane thickening, glomerulosclerosis, and tubulointerstitial fibrosis (Soulis-Liparota, *et al.*, 1995).

MG-induced hydroimidazolone increased selectively in retinas of streptozotocin-induced diabetic rats (Karachalias, *et al.*, 2003). In addition, MG-modified CML was localized in retinal blood vessels of patients with type II diabetes and was found to correlate with the degree of retinopathy (Stitt, 2001). Furthermore, decreased eNOS expression was observed in retinal vascular endothelial cells exposed to AGEs, which may account for retinal microvascular abnormalities (Chakravarthy, *et al.*, 1998).

MG-induced oxidative stress in diabetes mellitus

Growing evidence suggests that MG-induced oxidative damage is responsible for the development of diabetic complications. Type II diabetes mellitus patients without a history of acute cardiovascular events, such as myocardial infarction and unstable angina, during the previous 6 months were recruited. Compared to baseline, MG/AGEs rich, heat-processed food reduced macrovascular flow-mediated dilatation and decreased microvascular reactive hyperemia, indicating macro- and microvascular endothelial dysfunction. The impairment of postprandial flow-mediated dilatation may be the result of a combined effect of reduced NO production and increased NO scavenging, both decreasing NO bioavailability (Stirban, *et al.*, 2006).

Another study of three diabetic populations, the Overt Nephropathy Progressor / Nonprogressor (ONPN) cohort (n = 14), the Natural History of Diabetic Nephropathy study (NHS) cohort (n = 110) and the Pima Indian cohort (n = 45), demonstrated that progression of diabetic nephropathy was significantly correlated with MG levels and oxidative stress (Beisswenger, *et al.*, 2005). The oxidative stress in this study was verified by the reduced GSH levels in red blood cells of diabetic patients. In addition, MG modified renal mitochondrial protein in streptozotocin-treated rats, leading to significantly increased production of mitochondrial $O_2^{\cdot -}$ and oxidative damage (Rosca, *et al.*, 2005). Furthermore, exposure of human neuroblastoma SH-SY5Y cells to MG was associated with increased ROS production, leading to MG-induced cellular damage (de Arriba, *et al.*, 2006). MG also induced diabetic neuropathy through oxidative stress-mediated activation of p38 MAPK.

MG and insulin

Evidence shows that MG destroys pancreatic β-cells, decreases insulin secretion in response to glucose and alters insulin structure and function. MG caused a concentration-dependent increase of apoptotic pancreatic β-cells (Sheader, *et al.*, 2001). Addition of MG (0.5 or 1 mM) to single isolated rat pancreatic β-cells caused a rapid and marked depolarization, and this effect was reversible upon the removal of MG. MG also led to elevated cytosolic calcium concentration and intracellular acidification in intact rat islets (Cook, *et al.*, 1998). Moreover, acute exposure of isolated mouse islets or β-cells to MG resulted in reduced insulin secretion in response to glucose (Pi, *et al.*, 2007).

Immune alterations in diabetes

The damage of the immune system by MG glycation could be a link between diabetes and infection susceptibility (Claire and Stella, 2009). A commonly reported feature of diabetes is reduced production of the proinflammatory cytokine interferon-γ (IFN- γ) (Faresjo *et al.*, 2006). Type I diabetes and Type II diabetes patients also show inhibited tumour necrosis factor-α (TNF-α) production and reduced expression of T_H^1-associated chemokine receptors on peripheral blood cells (Lohmann *et al.*, 2002), as well as reduced production of the antiviral cytokine IFN-α from dendritic cells (DCs). Type I diabetes patients have lower proportions of natural killer (NK) cells in peripheral blood (important in clearance of virus-infected cells) and a reduced activity of these cells (Rodacki *et al.*, 2006) Diabetes is also associated with raised levels of serum adhesion factors, such as intercellular- and vascular-adhesion molecules (ICAM and VCAM) (Boulbou *et al.*, 2005; Glowinska *et al.*, 2005) and monocytes from type I diabetes patients show increased adhesion but decreased chemotactic migration towards proinflammatory chemokines (Bouma *et al.*,2005). The chemotactic, phagocytic and microbicidal functions of neutrophils are also hindered (Alba-Loureiro *et al.*,2007). Increased cell death is associated with hyperglycaemia, with greater apoptosis of lymphocytes and neutrophils reported in type I diabetes and type II diabetes patients (Otton *et al.*, 2004; Glisic-Milosavljevic *et al.*, 2007). Loss of these cell types might hinder both innate and adaptive responses to any infection.

Together, these studies illustrate that hyperglycaemia impacts many different facets of immune function. Any one aspect of these diabetes-associated deficits, including reduced leukocyte migration, impaired killing mechanisms or lower lymphocyte proliferation, might be enough to suppress infection clearance. MG appears to be a potent modifier of immune function in vitro and seems to be able to produce effects which confirm some of the in vivo findings described in diabetes patients. Reduced stimulation of T cells, altered cell phenotype and hindered cytokine responses caused by MG might underlie reduced infection clearance in diabetes.

Free radicals and diabetes mellitus

Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids and eventually cell death. Various mechanisms have been suggested to contribute to the formation of these reactive oxygen-free radicals. Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Wolff and Dean, 1987; Jiang *et al.*, 1990). Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals (Halliwell and Gutteridge, 1990; Hogg *et al.*, 1993). Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Kawamura *et al.*, 1994; Tsai *et al.*, 1994). Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and then advanced glycation endproducts (AGEs) (Mullarkey *et al.*, 1990; Hori *et al.*, 1996). Similar reactions are also found to occur with nucleic acids and their bases where glucose and other glycating agent reacts with free amino groups of guanine and less common to adenine to form AGEs, hence also called DNA-AGEs. These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions (McCarthy *et al.*, 2001), promote free radical formation (Baynes and Thorpe, 1999), and quench and block anti proliferative effects of nitric oxide (Wautier *et al.*, 1994; Vlassara,

1997). By increasing intracellular oxidative stress, AGEs activate the transcription factor NF-κB, thus promoting up-regulation of various NF-κB controlled target genes (Mohamed *et al.*, 1999). NF-κB enhances production of nitric oxide, which is believed to be a mediator of islet beta cell damage. Considerable evidence also implicates activation of the sorbitol pathway by glucose as a component in the pathogenesis of diabetic complications, for example, in lens cataract formation or peripheral neuropathy (Kador *et al.*, 1984; Greene *et al.*, 1992; Obrosova *et al.*, 1997). Efforts to understand cataract formation have provoked various hypotheses. In the aldose reductase osmotic hypothesis, accumulation of polyols initiates lenticular osmotic changes. In addition, oxidative stress is linked to decreased glutathione levels and depletion of NADPH levels (Cheng and Gonzalez, 1986). Alternatively, increased sorbitol dehydrogenase activity is associated with altered NAD^+ levels (Williamson *et al.*, 1993), which results in protein modification by nonenzymatic glycosylation of lens proteins (Yano *et al.*, 1989; Ramalho *et al.*, 1996). Mechanisms linking the changes in diabetic neuropathy and induced sorbitol pathway are not well delineated. One possible mechanism, metabolic imbalances in the neural tissues, has been implicated in impaired neurotrophism (Mizisin *et al.*, 1997; Delcroix *et al.*, 1998; Hounsom *et al.*, 1998), neurotransmission changes (Ralevic *et al.*, 1995; Kamei and, Ohsawa, 1996; Stevens *et al.*, 2000), Schwann cell injury (Mizisin *et al.*, 1998; Kalichman *et al.*, 1998) and axonopathy (Chokroverty *et al.*, 1988; Fernyhough *et al.*, 1999).

Epidemiology and prevalence of diabetes mellitus

The worldwide prevalence of diabetes mellitus has risen dramatically over the past two decades, from an estimated 30 million cases in 1985 to 177 million in 2000 (Fig. 2). Based on current trends, > 360 million individuals will have diabetes by the year 2030 (Wild *et al.*, 2004). Estimates which have been produced by the International Diabetes Federation (IDF) is about 194 million individual worldwide to have diabetes in 2003 and is expected to increase to about 333 million by 2025. (International Diabetes Federation, Diabetes Atlas, 2006).

Table 3 shows the 10 countries with the largest numbers of people with diabetes. As might be expected, the countries with the largest populations have

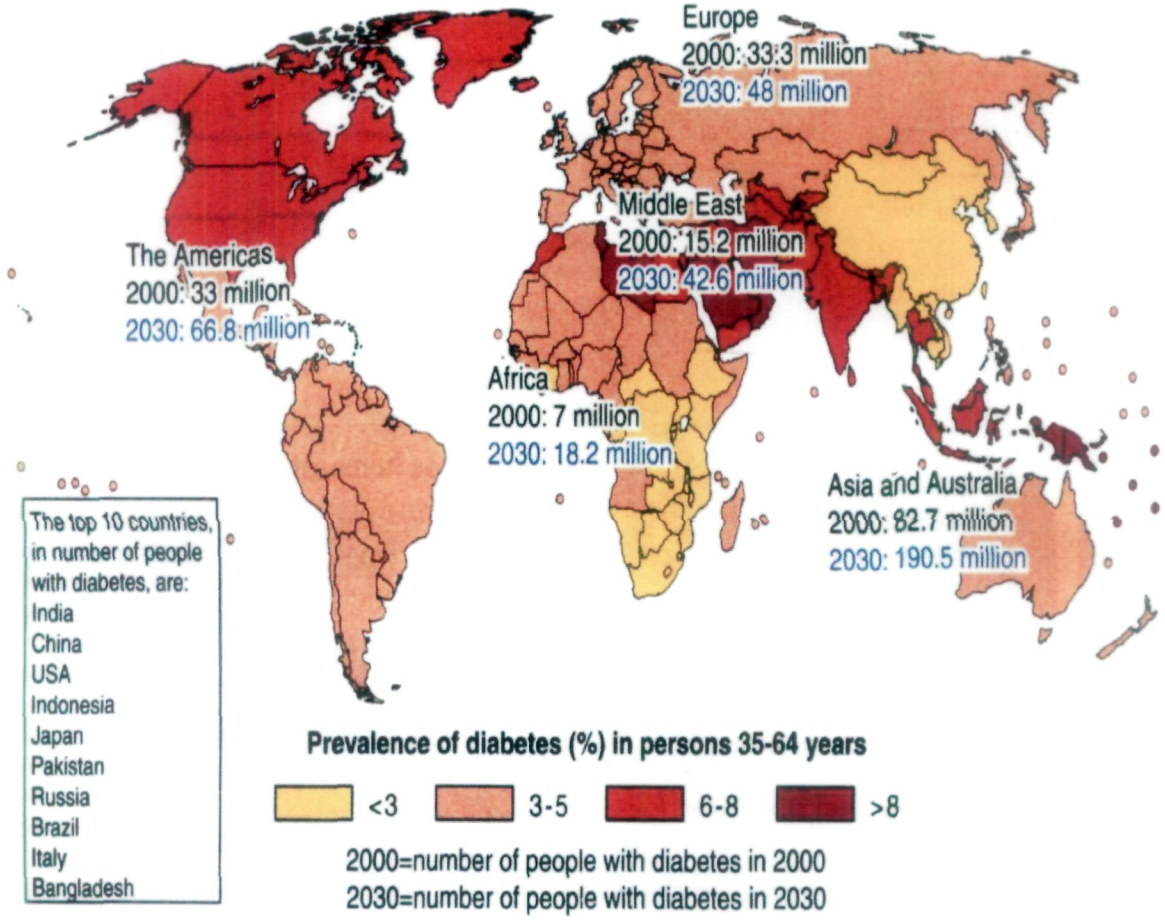


Fig. 2 **Worldwide prevalence of diabetes mellitus.**

Source: Adapted from Wild, S. *et al*, diabetes care (2004) **27**, 2004.

TABLE 3

Top Ten countries for numbers of people aged 20–79 years with diabetes in 2010 and 2030. (Number of diabetic peoples, *in millions*).

S. No.	Country	Year, 2010	S. No.	Country	Year, 2030
1	India	50.8	1	India	87.0
2	China	43.2	2	China	62.6
3	USA	26.8	3	USA	36.0
4	Russia	9.6	4	Pakistan	13.8
5	Brazil	7.6	5	Brazil	12.7
6	Germany	7.5	6	Indonesia	12.0
7	Pakistan	7.1	7	Mexico	11.9
8	Japan	7.1	8	Bangladesh	10.4
9	Indonesia	7.0	9	Russian	10.3
10	Mexico	6.8	10	Egypt	8.6

Source: Shaw *et al.*, diabetes research and clinical practice **87**, 2010

the highest number of persons with diabetes. Only Bangladesh and Nigeria of the world's 10 most populous countries are not among the 10 countries with the highest diabetes numbers (replaced by Germany and Mexico) for 2010. There are marked differences between developed and developing countries. For developing countries, adult diabetes numbers are likely to increase by 69% from 2010 to 2030, compared to 20% for developed countries, whereas total adult populations are expected to increase by 36% and 2% respectively (Shaw *et al.*, 2010).

There is considerable geographic variation in the incidence of both type I and type II diabetes mellitus. Scandinavia (Finland) has the highest incidence of type I diabetes mellitus, whereas in Pacific Rim regions (Japan, China), its prevalence is least. Northern Europe and the United States have an intermediate rate. Much of the increased risk of type I diabetes mellitus is believed to reflect the frequency of high risk HLA alleles among ethnic groups in different geographic locations. The prevalence of type II diabetes mellitus and its harbinger, IGT, is highest in certain pacific islands, intermediate in countries such as India and the United States, and relatively low in Russia. This variability is due to genetic, behavioural and environmental factors.

India is now being termed the "diabetes capital of the world" as it leads the world with largest number of diabetic subjects. WHO report shows that 32 million people in India had diabetes in the year 2000 and is expected to increase about 80 million by 2030. The IDF estimated the total number of diabetes subjects to be around 36 million in India in 2003 and this is further set to rise to 73.4 million by the year 2025. Another report published recently, estimated it to be 50.8 million by the year 2010 and will shoot up to 87.0 million by the end of year 2030 (Shaw *et al.*, 2010).

Diabetes is the leading cause of mortality. A recent estimate suggested that diabetes is the fifth leading cause of death worldwide and is responsible for almost 3 million deaths annually, which is a 1.7-5.2% of deaths worldwide.

Objective

Recently it has been demonstrated that like proteins, DNA is susceptible to nonenzymatic attack by sugar affecting the structure, stability and conformation of DNA molecule. Although the nonenzymatic glycation of biomolecules occurs naturally, in some metabolic disorders such as diabetes, the formation and accumulation of AGEs occurs faster. This rapid formation and accumulation of AGEs is caused not only by D-glucose itself, but also by certain glucose-derived dicarbonyl intermediates such as methylglyoxal (MG), which is a potent precursor of AGEs. The highly reactive electrophile MG, a break down product of carbohydrate is a major environmental mutagen, an off-shoot product of glycolysis which is generated from deprotonation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. It is also produced from lipid peroxidation and acetone metabolism. Additional endogenous sources include catabolism of threonine and oxidative breakdown of DNA and RNA. MG mutagenicity has been reported in *Escherichia Coli*, wherein it causes mutations at G:C base pairs, as well as in *Sacharomyces cerevisiae*. It has been suggested that MG reacts with free amino groups of proteins and DNA under physiological conditions and forms advanced glycation end products resulting in the generation of hydroxyl ($^{\cdot}OH$) and superoxide ($O_2^{\cdot-}$) ions which play an important role in the pathophysiology of ageing and diabetic complications. Methylglyoxal reacts with 2'-deoxyguanosine via the classic amadori pathway and did not react appreciably with 2'-deoxyadenosine, 2'-deoxythymidine and 2'-deoxycytidine. However, reaction of double stranded DNA or guanine/ 2'-deoxyguanosine with MG *in-vitro* produces primarily N^2-carboxyethyl-2'-deoxyguanosine (CEdG), as a major adduct. This adduct (CEdG) might be used as a useful biomarker for monitoring oxoaldehyde-induced stress in response to enhance glycolytic flux or environmental exposure to MG.

In the present study commercially available human placental DNA has been modified by MG and lysine in the presence and absence of Cu^{2+}. The modifications on DNA were studied by ultraviolet, fluorescence and circular dichroism spectroscopy, thermal denaturation studies, nuclease-S1 digestibility, HPLC, LC-MS and ESI-MS. Comet assay was performed to detect alkali labile sites, single and double stranded DNA breaks on human lymphocytes exposed to MG alone, lysine alone and in combination of MG + lysine and MG + lysine + Cu^{2+}. The hydroxyl

($^{\cdot}OH$) and superoxide ($O_2^{\cdot-}$) radicals generated in MG + lysine + Cu^{2+} system have been also quantitated. Polyclonal antibodies against native and MG-Lys-Cu^{2+} modified human placental DNA have been generated in experimental animals. The specificity of induced antibodies has been evaluated by competition ELISA and gel retardation assay. These antibodies have been used as an immunochemical probe to detect MG-Lys-Cu^{2+} induced damage in the DNA, isolated from diabetic patients. In order to assess the possible role of MG-Lys-Cu^{2+} modified DNA in the etiology of type I diabetes mellitus, sera from diabetes patients have been assessed for their binding to native and MG-Lys-Cu^{2+} modified human placental DNA. Furthermore, binding of serum antibodies from type II diabetes patients with native and MG-Lys-Cu^{2+} modified human placental DNA has been also evaluated.

Materials and Methods

Materials

Chemicals

Methylglyoxal (MG), thiobarbituric acid (TBA), 2 deoxy-D-ribose, cytochrome-C, catalase, nuclease S1, anti-human and anti-rabbit IgG alkaline phosphatase conjugates, methylated bovine serum albumin (MBSA), p-nitrophenyl phosphate, superoxide dismutase (SOD), ethidium bromide, Coomassie Brilliant Blue G-250, sodium dodecyl sulphate, Tween-20, Protein A-agarose (2.5ml pre-pack column), Freund's complete & incomplete adjuvants, agarose, proteinase K, mannitol, histopaque 1077, low melting point agarose (LMPA), sodium azide and dialysis tubing were purchased from Sigma Chemical Company, U.S.A. Pyridoxal phosphate (PLP) was from Calbiochem, U.S.A. Dihydroxy acetone (DHA) was from Merck, Germany. Ficoll 400 and xylene cyanole FF were purchased from Pharmacia Fine chemicals, Sweden. Lysine was from Sisco research laboratory. Triton X-100 was procured from Hi-Media. Trizma base was from Spectrochem, Mumbai, India. ELISA plates (96 wells) were purchased from NUNC, Denmark. Acrylamide, bisacrylamide, ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratories, U.S.A. EDTA, (disodium salt), silver nitrate, dimethyl sulphoxide (DMSO), chloroform, D-mannitol, methanol, glacial acetic acid, iso-propanol, sodium chloride, sodium carbonate, sodium nitrite, sodium hydroxide, sodium bicarbonate, copper sulphate, magnesium chloride, trichloro acetic acid, manganese dioxide and potassium chloride were from Qualigens, India. Diphenylamine and ethanol were chemically pure. All other reagents/chemicals were of the highest analytical grade available.

Equipments

Shimadzu UV-1700 Spectrophotometer equipped with a thermo-programmer and controller unit, Spectrofluorometer (Shimadzu, RF-5301), Spectropolarimeter (Jasco J-815), ELISA microplate reader (Labsystem Multiskan EX, FINLAND), ELICO pH meter (L1-120), UV-transilluminator (Vilber Lourmat, France), Agarose and polyacrylamide gel electrophoresis assemblies (Genei, India), Avanti 30 table top high speed refrigerated centrifuge (Beckman, USA). Gel-doc (Bio-Rad laboratories, U.S.A), Agilent 1100 HPLC system (Palo Alto, California) and C-18 column

(Synergi, U.S.A), an orthogonal time of flight (TOF) mass spectrometer (Applied Biosystems, Mariner atmospheric pressure ionization TOF workstation, Framingham, MA, USA) equipped with Standard ESI source and LC-MS; Micromass Quattro II triple quadrupole mass spectrometer (Beverly, MA) coupled with HPLC were the major equipments used in this study.

Collection of sera

Fasting blood samples of diabetic patients, both type I and II were obtained from J.N. Medical College Hospital, A.M.U, Aligarh. None of the diabetic patients had other autoimmune diseases. Specifically, there were no cases of lupus or other connective tissue disorders. Normal human sera were obtained from healthy subjects. Samples were collected in a glass test tube and left to clot for 30 min at 37°C. Serum was separated by centrifugation at 3000 rpm for 10 min. Serum samples were heated at 56°C for 30 min to inactivate complement proteins and stored in aliquots at –20°C with 0.1% sodium azide as preservative.

Methods

Purification of human placental DNA

Commercially available human placental DNA was purified free of proteins and single stranded regions (Ali *et al.*, 1985). DNA (2 mg/ml) was dissolved in 0.1 X SSC buffer, pH 7.3 and extracted with an equal volume of chloroform-isoamyl alcohol (24:1) in a stoppered container and shaken for 1 hr. The aqueous layer containing DNA was separated from organic layer and re-extracted with chloroform-isoamyl alcohol mixture. DNA was precipitated with two volumes of chilled absolute ethanol and collected on a glass rod. After drying in air, the DNA was dissolved in acetate buffer (30 mM sodium acetate containing 30 mM zinc chloride, pH 5.0) and treated with nuclease S1 (150 units/mg DNA) at 37°C for 30 min to remove single stranded regions. The reaction was stopped by adding one-tenth volume of 200 mM EDTA, pH 8.0. Nucleases S1 treated DNA was extracted twice with chloroform-isoamyl alcohol and finally precipitated with two volumes of chilled ethanol. The precipitate was dissolved in the required buffer.

Determination of DNA concentration

DNA concentration was estimated colorimetrically by the method of Burton (1956) using diphenylamine reagent. This assay depends upon a color reaction of deoxyribose. Exposure of DNA to strong acids removes the purine bases, leaving a polymer known as apurinic acid. With the release of purine, the exposed reactive aldehyde group of deoxyribose reacts with diphenylamine producing blue color, which serves as the basis for estimating DNA concentration.

Crystallization of diphenylamine

Diphenylamine (2g) was dissolved in 200 ml boiling hexane. After adding 0.5g of activated charcoal the hot mixture was filtered through Whatman filter paper (No.1) and the filtrate was kept overnight at 4°C. The preparation was finally dried at room temperature before use.

Preparation of diphenylamine regent

To 750 mg of recrystallized dipenylamine was added 50 ml of glacial acetic acid followed by 0.75ml concentrated sulphuric acid. The regent was prepared fresh before use.

Procedure

Varying amounts of DNA in 1.0 ml of assay volume was mixed with 1.0 ml of 1N perchloric acid and incubated at 70°C for 15 min. One hundred microlitre of 5.43 mM acetaldehyde was added followed by 2.0 ml of freshly prepared diphenylamine reagent. The contents were mixed and incubated at room temperature for 16-20 hr for colour development. Absorbance was read at 600 nm and DNA concentration in unknown samples was determined from the standard plot of purified calf thymus DNA.

Determination of protein concentration

Protein was estimated by the methods of Lowry *et al.* (1951) and Bradford (1976).

Protein estimation by Folin's-Phenol reagent

Protein estimation by this method utilizes alkali (to keep the pH high), Cu^{2+} ions (to chelate proteins) and tartarate (to keep the Cu^{2+} ions in solution at high pH).

(a) Folin-Ciocalteau reagent

The Folin-Ciocalteau reagent was diluted 1:4 with distilled water before use.

(b) Alkaline copper reagent

The components of alkaline copper reagent were prepared as under:

(i) Two percent sodium carbonate in 100 mM sodium hydroxide.
(ii) 0.5 percent copper sulphate in 1.0 percent sodium-potassium tartarate.

The working reagent was prepared fresh by mixing components (i) and (ii) in 50:1 ratio.

(c) Procedure

Varying amounts of protein in 1.0 ml of volume was mixed with 5.0 ml of freshly prepared alkaline copper reagent and incubated at room temperature for 10 min. One ml of working Folin-Ciocalteau reagent was added followed by 30 min incubation at room temperature. The absorbance was monitored at 660 nm. Protein content of unknown sample was determined from the standard plot of bovine serum albumin.

Protein estimation by dye-binding method

This assay is based on the change in absorption maxima of an acidic solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm when protein binds to the dye. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

Dye preparation

One hundred mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid added. The resulting solution was diluted to a final volume of 1 litre and filtered through whatman filter paper (No.1) to remove the undissolved particles.

Protein assay

To one ml of solutions containing 10-100 mg protein was added 5 ml of dye solution and contents were mixed by vortexing. The absorbance was read at 595 nm after 5 min against a reagent blank.

Modification of human placental DNA by methylglyoxal (MG) and lysine in presence of Cu^{2+}

Human placental DNA was modified by methylglyoxal and lysine in the presence and absence of Cu^{2+} as described by Kang (2003) with slight modification. 37.8 μM of human DNA was thoroughly mixed with methylglyoxal (40 mM), lysine (40 mM) and Cu^{2+} (300 μM) in 10 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl and incubated at 37°C for 24 hr. followed by extensive dialysis against PBS to remove unbound constituents.

Spectroscopic analysis

The ultraviolet absorption profile of native human placental DNA and modified DNA was recorded in the wavelength range of 200-400 nm on Shimadzu UV-1700 spectrophotometer.

Fluorescence analysis

Fluorescence emission profile of native human placental DNA and modified DNA were recorded on Shimadzu RF-5301 spectrofluorometer. Native and modified DNA were excited at 370 nm and emission profile was recorded at 450 nm.

Circular dichroism measurements

Circular dichroism (CD) profile of native human placental DNA and modified human placental DNA were recorded on spectropolarimeter (Jasco J-815) in a 1 cm path length cell at 25°C. The wavelength range was from 220 nm to 400 nm and all the scans were recorded at an interval of 0.2 nm. The base line was corrected with PBS, pH 7.4 and the DNA was used at 37.8 μm concentration. Molar ellipticities [θ] were calculated in terms of base pair concentration according to the following equation:

$$[\theta] = \frac{\theta}{10cd}$$

where, [θ] represents measured ellipticity in mdeg, 'c' is the molar concentration of human placental DNA per base pair and 'd' is the path length.

Thermal denaturation studies

Thermal denaturation analysis of DNA was performed to ascertain the thermal stability of DNA constituent to modification. Mid point melting temperature (Tm), of native and modified DNA samples was determined by subjecting them to heat denaturation on Shimadzu UV-1700 Spectrophotometer equipped with a thermo-programmer and controller unit (Habeeb *et al.*, 2005). All the samples were melted from 30°C to 95°C at a rate of 1.0 ^{0}C/min. The change in absorbance at 260 nm was recorded with increasing temperature.

Percent denaturation was calculated as per the following equation:

$$\text{Percent denaturation} = \frac{A_T - A_{30}}{A_{max} - A_{30}} \times 100$$

Where, A_T = Absorbance at a temperature T°C.

A_{max} = Final maximum absorbance on the completion of denaturation (95°C).

A_{30} = Initial absorbance at 30°C.

Measurement of superoxide anion

The generation of $O_2^{\cdot -}$ in the reaction mixture was determined by cytochrome-c reduction assay (Beauchamp and Fridovich, 1971). A reaction mixture contained MG (40 mM) and lysine (40 mM) and 100 μM cytochrome C in 10 mM phosphate buffer (pH 7.4). The reduction rate was determined as the increase in absorbance at 550 nm for 10 min at room temperature. Absorbance was taken at the interval of every one min.

Measurement of hydroxyl radical

Detection of hydroxyl radicals was carried out by measuring thiobarbituric acid (TBA) reactive 2-deoxy-D-ribose oxidation products (Halliwell and Gutteridge, 1981). Reaction mixtures containing 40 mM MG, 40 mM lysine in the presence and absence of 300 μM Cu^{2+} were incubated at 37°C for 24 hr. The degradation of 2-deoxy-D-ribose was measured by adding 1 mL of 2.8% (w/v) trichloroacetic acid, 1 mL of 1% (w/v) thiobarbituric acid followed by heating at 100°C for 10 min. After cooling the absorbance was read at 532 nm. Because a generation of TBARS could be induced by the reaction of MG with lysine in absence of deoxyribose, the reaction mixture containing MG and lysine was used as the blank.

Detection of strand breaks

The strand breaks generated as a result of modification of human placental DNA by MG, Lys and Cu^{2+} were detected by nuclease S1 digestibility assay.

Gel preparation

Agarose 0.8% in TAE buffer (40mM Tris-acetate, pH 8.0 containing 2 mM EDTA) was dissolved by heating. The solution was cooled to about 50 °C and then poured into the gel tray and allowed to solidify at room temperature.

Agarose gel electrophoresis

Native and modified human placental DNA were mixed with 1/10 volume of sample buffer (0.125% bromophenol blue, 30% Ficoll-400, 5 mM EDTA in 10 X

electrophoresis buffer) and loaded in the wells of 0.8% agarose gels. Electrophoresis was done for 2 hr at 40 mA and the gels were stained with ethidium bromide (0.5 μg/ml), and viewed under UV light.

Nuclease S1 digestibility

Native human placental DNA and the modified analogue were characterized by nuclease S1 digestibility assay (Matsuo and Ross, 1987). One μg each of native DNA and the modified DNA in acetate buffer (30 mM sodium acetate, 1 mM zinc sulphate, 100 mM sodium chloride, pH 4.6) were treated with nuclease S1 (20 unit/μg of DNA) for 30 min at 37°C. The reaction was stopped by adding one tenth volume of 200 mM EDTA, pH 8.0. The samples were subjected to agarose gel electrophoresis and viewed by illumination under UV light after ethidium bromide (0.5 μg/ml) staining.

Synthesis of the standard, carboxyethyl deoxy guanosine (CEdG)

The synthesis of CEdG was carried out as described by Seidel and Pischetsrieder, (1998) with slight modifications. Briefly, 50 mg of deoxy-guanosine suspended in 1ml of 100 mM phosphate buffer (pH, 7.4), was incubated with 100 mg of dihydroxyacetone at 70°C in a shaking water bath. It got dissolved at 70°C in the course of reaction after 24 hr. CEdG was isolated by preparative HPLC using 50 mM ammonium acetate buffer solution and methanol as eluents.

^{1}H NMR of the standard CEdG

^{1}H NMR spectra were recorded on a Bruker DRX-300 MHz FT NMR spectrometer. All samples of NMR spectroscopy were first lyophilized and prepared using DMSO as a soluent. The chemical shifts in parts per million (ppm) are expressed with respect to tetra methyl silane (TMS) as a reference.

Detection of a glycated product, CEdG in modified human placental DNA by HPLC and LC-MS

An Agilent 1100 capillary HPLC System equipped with a Synergi C_{18} analytical column was used for HPLC analysis of native and modified analogue of human placental DNA. General chromatographic conditions were as follows:

C_{18} column (2 mm × 150 mm with 4 μm particle size); eluent A, 5 mM aqueous ammonium acetate buffer, pH 7; eluent B, Acetonitrile (CH_3N) gradient solution- the CH_3N concentration was raised from 0 to 4.0% in the first 5 min; from 4.0 to 6.5% over 30 min; held at 6.5% for 5 min, and then raised to 90% to wash residual material off the column at a constant flow rate of 500 μL/min. DNA bases were detected by DAD at 254 nm, their absorption maximum. LC- MS analyses of CEdG standard were carried out using a Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer interfaced to an Agilent 1100 capillary HPLC system.

Characterization of DNA-AGEs by electrospray ionization mass spectrometry

An orthogonal time of flight (TOF) mass spectrometer (Applied Biosystems Mariner Atmospheric Pressure Ionization TOF Workstation, Framingham, MA, USA) equipped with standard electrospray ionization source was used. The mass spectral data were collected at positive ion polarity. Nitrogen was used as the nebulizer, heater and collision gas. The sciex heater was set to 350°C and the spray tip potential was set at 4,000 V. The instrument was outfitted with an integrated syringe pump with a dual syringe rack for direct infusion onto the mass spectrometer. The mass spectrometry system was operated on full scan mode (m/z 100-1000). Spectral acquisition was performed every 2 s and a total of ten spectra were accumulated. The final spectrum depicts an average of 4-6 scans.

Quenching studies

Quenching studies were preformed to ascertain the type of radical causing damage to the human placental DNA. The effect of various quenchers like catalase (50 units/ml), superoxide dismutase (SOD, 600 units/ml), hydroxyl radical scavengers (D-mannitol 50 mM each), nitric oxide trapping agent (Carboxy-PTIO at 25 mM), antioxidants (uric acid at 0.1 mM) and metal ion chelator (EDTA at 20 mM) on human placental DNA modification were studied by incubating DNA 25 μg/ml in 10 mM sodium phosphate buffer, pH 7.4 containing MG (40 mM), lysine (40 mM) and Cu^{2+} (300 μm) at 37°C for 24 hr. Percent quenching was calculated from absorbance at A_{260} nm.

AGE inhibition study

AGE inhibition was performed using a carbonyl trapping compound, D-Penicillamine (1 mM) and pyridoxal phosphate (10 mM) on human placental DNA. The modifications were studied by incubating DNA 25 μg/ml in 10 mM sodium phosphate buffer, pH 7.4 containing MG (40 mM), lysine (40 mM) and Cu^{2+} (300 μM) at 37°C for 24 hr. Percent DNA quenching was calculated from absorbance at A_{260} nm. AGE inhibition was further confirmed from absorbance at A_{330} nm.

Genotoxicity of advanced glycation endproducts by single cell gel electrophoresis (SCGE); comet assay

Lymphocyte isolation

Blood was obtained from a healthy male volunteer (myself). Lymphocytes were separated from heparinised whole blood. Briefly, 3 ml of blood was drawn through venous puncture and added to 3 ml of PBS and layered over 2 ml of histopaque 1077 and centrifuged at 800 X g for 2 min. The visible 'buffy' coat was aspirated into 3-5 ml of PBS and centrifuged at 250 X g for 10 min to pellet the lymphocytes. The pellet was resuspended in 1 ml of RPMI media and counted over a Haemocytometer. Approximately 10,000 cells per 100 μl of medium are taken for each dose of the test material.

Alkaline comet assay

Comet assay was performed with human lymphocytes following the method of Singh *et al.* (1998) with slight modification. Freshly isolated cells were treated with Lysine (40 mM) only, MG (40 mM) only, and in combination of MG + Lysine (40 mM each) and MG + Lysine (40 mM each) + Cu^{2+} (300 μM) for 24 hours at 37°C. Untreated lymphocytes served as control. The lymphocytes ~10,000 cells, both treated and untreated, were suspended in 100 μl of Ca^{2+} and Mg^{2+} free Dulbecco's PBS (1 L packet: add 990 mL dH2O, adjust pH to 7.4 and make up to 1 L) and mixed with 100 μl of 1% LMP agarose. The cell suspension (80 μl) was then layered on one third frosted slides, pre-coated with 1% normal agarose and kept at 4°C for 10 min. After gelling, a layer of 90 μl of 0.5% LMP agarose was added. The cells were then

lysed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, pH 10.0 containing 1% Triton X-100) for overnight. After washing with MQ water, the slides were subjected to DNA denaturation in cold electrophoretic buffer (300 mM NaOH/ 1 mM EDTA, pH 10.0) at 4°C for 20 min. Electrophoresis was performed at 300 mA, 24 V (0.74 V/cm) at 4°C. The slides were then washed three times with neutralization buffer (0.4 M Tris, pH 7.5). All preparative steps were conducted in dark to prevent secondary DNA damage. The slides were stained with ethidium bromide (10 μg/ml) for 5 min and covered with coverslip.

Analysis of cells

A total of 50 cells from each slide were selected randomly and analyzed by image analysis using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. Liverpool, UK. Observations were made at 400 X magnifications using an epifluorescent microscope linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percent DNA in tail. Therefore, percentage of tail DNA represents the average of total 50 individual cells. The three independent readings for each sample were averaged further to arrive at a final value for each sample. The mean value of all the 5 samples was averaged to get the average for each treatment as well as control also.

Isolation of DNA from lymphocytes

Human DNA was isolated from lymphocytes of normal and diabetic patients as described by Ashok and Ali (1998). The lymphocytes were lysed by lysis buffer (10 mM Tris, 2 mM EDTA, 400 mM NaCl, containing 1% SDS and 1mg/ml proteinase K, pH 7.4)

Procedure

The lymphocytes were resuspended in lymphocyte lysis buffer, incubated at 50°C for 1 hr and overnight at 37 °C with constant shaking. Equal volume of chloroform-isoamyl alcohol (24:1) was added and the sample extracted for 15 min and left to stand for 30 min. The aqueous layer was removed carefully and the

extraction step was repeated 2-3 times. To the aqueous layer was added one-tenth volume of 3 N sodium acetate, pH 5.2 and 2 volumes of cold absolute ethanol to precipitate DNA. The DNA was collected by centrifugation, washed once with 70% ethanol, allowed to dry and subsequently dissolved in PBS, pH 7.4. Absorbance of DNA solution was monitored at 260 nm and 280 nm to ascertain its purity and concentration.

Immunization schedule

Female rabbits weighing 1- 1.5 Kg were immunized in duplicates with native and modified human placental DNA (50 μg each) respectively. DNA samples were complexed with equal amount of methylated BSA in 1:1 ratio (w/w) emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly at multiple sites of the animal. Booster injections were given in Freund's incomplete adjuvant at weekly intervals. During the course of immunization, each animal received 350 μg antigen. Seven days post boost test bleeds were used to find out the titre of induced antibodies. Marginal ear vein of immunized animals was punctured and blood was carefully collected to prevent hemolysis. Serum was separated and complement proteins were inactivated by heating at 56°C for 30 min. Pre-immune blood was collected prior to immunization. The sera were stored in small aliquots at -20°C with 0.1% sodium azide as preservative.

Isolation of IgG on protein A-agarose

Serum IgG was isolated by affinity chromatography on Protein A-agarose affinity column. Serum (0.5ml) diluted with equal volume of PBS, pH 7.4 was applied to column (12 mm × 45 mm) equilibrated with the same buffer. The wash through was recycled 2-3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding, 1976) and neutralized with 1 ml of 1M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 251 and 278 nm. IgG concentration was determined considering 1.40 OD_{280}= 1.0 mg mammalian IgG/ml.

SDS-Polyacrylamide gel electrophoresis

The homogeneity of purified IgG was ascertained by polyacrylamide gel electriphoresis under non-reducing conditions (Laemmli, 1970). The following stock solutions were prepared.

Acrylamide-bisacrylamide (30:0.8)

A stock solution was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in distilled water to a final volume of 100 ml. The solution was filtered and stored at 4°C in an amber colored bottle.

Resolving gel buffer

A stock solution was prepared by dissolving 36 gm Tris base in 48 ml of 1.0 N HCI, pH was adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

Electrode buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH was adjusted to 8.3 and volume adjusted to one litre.

Procedure of PAGE

Glass plates separated by 1.5 mm thick spacer were sealed with 1% agarose from sides and bottom. The non-reducing gel was prepared, poured between the glass plates and allowed to polymerize at room temperature.

Protein sample (IgG) was mixed with one-fourth volume of sample buffer (10% glycerol and 0.002% bromophenol blue in 10 times concentrated 0.5 M Tris-HC1, pH 6.8) and loaded into the well for electrophoresis at 70 volts for 4 hr in Tris-glycine buffer. The gels were stained with silver nitrate, which involved fixing the protein bands for 10 min in a mixture of 40% methanol and 13.5% formaldehyde followed by 2x washing with distilled water, at an interval of 5 min. Then the gel was immersed in 0.02% sodium thiosulphate solution for 1 min followed by two washings

with distilled water at an interval of 20 sec. The gel was immersed in 0.1% silver nitrate solution for 10 min and finally washed with distilled water.

The gel was then immersed in the developer (3% sodium carbonate, 2% sodium dithiosulphate and 0.05% formaldehyde) to facilitate staining and on appearance of visible bands, the reaction was stopped by a stopper solution (10% acetic acid and 10 % methanol).

Recipe for 7.5% SDS-PAGE

Solution	Stacking Gel	Resolving gel
Acrylamide-bisacrylamide	1.25 ml	7.5 ml
Stacking gel buffer	2.5 ml	-
Resolving gel buffer	-	3.75 ml
Distilled water	5.65 ml	16.95 ml
10% SDS	0.1 ml	0.3 ml
1.5% ammonium persulphate	0.5 ml	1.5 ml
TEMED	0.75 µl	15 µl

Immunological detection of antibodies

Sera were tested for antibodies by enzyme linked immunosorbent assay and gel retardation assay.

Enzyme linked immunosorbent assay

Following reagents were prepared and used in ELISA,

Tris buffered saline (TBS): 10 mM Tris, 150 mM NaCl, pH 7.4.

Tris buffered saline containing Tween 20 (TBS-T): 20 mM Tris, 144 mM NaCl, 2.68 mM KCl, pH 7.4 containing 500 µL Tween 20 per litre.

Carbonate-bicarbonate buffer: 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6 containing 2 mM magnesium chloride.

Substrate: 500 µg p-nitrophenyl phosphate (PNPP) per ml of carbonate-bicarbonate buffer, pH 9.6.

Procedure

ELISA was carried out on flat bottom polystyrene plates as described earlier (Ali and Alam, 2002). Briefly, microtitre wells were coated with one hundred microlitre of 2.5 μg/ml of DNA (in TBS, pH 7.4) and incubated for 2 hr at 37°C and overnight at 4°C. Each sample was coated in duplicate and half of the plate, devoid of antigen, served as control. The test-plate wells were emptied and washed thrice with TBS-T to remove the unbound antigen. Unoccupied sites were blocked with 150 μl of 1.5 % non-fat dry milk (in TBS, pH 7.4) for 4-5 hr at 4°C followed by single wash with TBS-T. In direct binding ELISA, antibodies were directly added into antigen-coated wells and incubated for 2 hr at 37°C and overnight at 4°C respectively. The wells were emptied and extensively washed with TBS-T. Anti-immunoglobulin G alkaline phosphatase conjugate was added to each well and incubated at 37°C for 2 hr and then the plates were washed thrice with TBS-T followed by a single wash with distilled water. Para-nitrophenyl phosphate was added and the developed color was read at 410 nm on a microplate reader. The results were expressed as mean of difference of absorbance values in test and control wells ($A_{test} - A_{control}$).

Competition ELISA

The specific binding characteristics of antibodies were ascertained in competitive binding assay. Varying amounts of inhibitors (0-20 μg/ml) were mixed with constant amount of antiserum or IgG. The mixture was incubated at room temperature for 2 hr and overnight at 4°C. Immune complex thus formed was coated in the wells instead of the serum. The remaining steps were the same as in direct binding ELISA.

Percent inhibition was calculated using the formula:

$$\text{Percent inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100$$

Gel retardation assay

Antigen-antibody specificity was further confirmed by the gel retardation assay. A constant amount of DNA antigen (1 μg) was mixed with varying amounts of

IgG (Habeeb *et al.*, 2005) and incubated for 2 hr at 37°C and overnight at 4°C. At the end of incubation, one-tenth volume of sample buffer was added to antigen-antibody complex and electrophoresed on 1% agarose gel in TAE buffer (pH 7.9) for 2 hr at 30 mA current. The gels were stained with ethidium bromide (0.5 mg/ml) and visualized under UV light and photographed.

Results

Ultraviolet absorption spectral studies of native and MG-Lys-Cu^{2+} glycated human DNA

Pilot experiments were undertaken to work out the time of incubation and optimum concentration of MG, lysine and Cu^{2+} needed to modify the DNA. Human DNA (37.8 μM) was incubated with MG, lysine and Cu^{2+} along with respective controls for different time intervals (3, 6, 12 and 24 hr) at 37 ^{o}C. Maximum hyperchromicity at 260 nm was obtained at 24 hr incubation mixture and further incubation did not result in any change in the hyperchromicity. Therefore, for further characterization, human DNA was incubated for 24 hr with 40 mM each of methylglyoxal and lysine in presence and absence of copper sulphate (Cu^{2+}, 300μM) in phosphate buffer saline was used. Native and modified DNA samples were subjected to UV- spectroscopical analysis on UV-1700 spectrophotometer. A characteristic peak at 260 nm was observed with native DNA (Fig. 3). However, upon modification with methylglyoxal + lysine (in absence of Cu^{2+} ions), 68% hyperchromicity was observed in comparison to native human DNA. While DNA modified with methylglyoxal + lysine (in presence of Cu^{2+}) exhibited further increase in hyperchromicity, which was 76% above that of native DNA. Furthermore, a new peak, more of a shoulder, appeared at 330 nm in the modified samples. This might be due to glycated nitrogenous bases of DNA. The controls, Cu^{2+}, lysine, lysine + Cu^{2+}, MG, and MG + Cu^{2+} treated DNA did not show appreciable hyperchromicity under similar conditions.

Agarose gel electrophoresis of native and MG-Lys-Cu^{2+} glycated human DNA

Electrophoresis of native and modified DNA was performed on 0.8% agarose gel. The gel pattern (Fig. 4) shows an increase in the mobility of glycated DNA with increasing incubation time (lanes 2- 5). Maximum mobility was observed at 24 hr incubation, and further incubation did not have any consequential effect on DNA migration pattern. The increase in mobility may be due to the generation of single stranded breaks by glycation induced intermediates which may cause the formation of small size DNA having faster mobility compared to native DNA of lane 1.

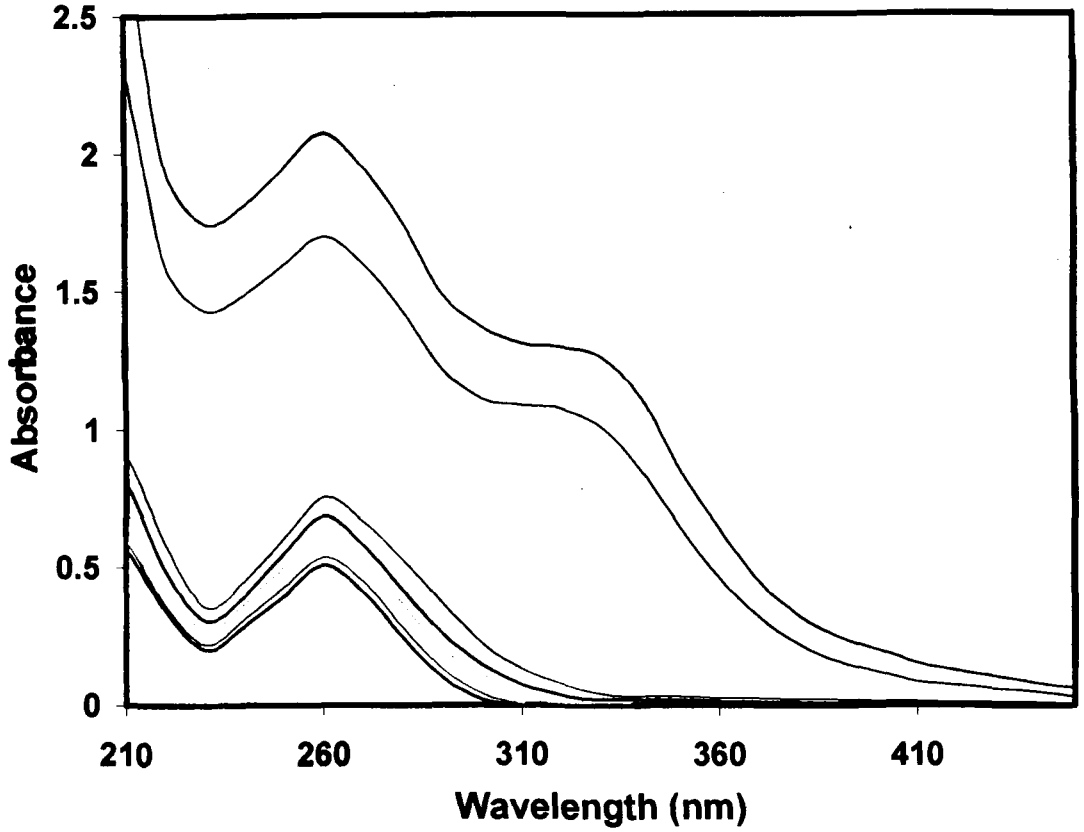


Fig. 3 Ultraviolet absorption spectra of native human DNA (–); human DNA modified with 300 μM Cu^{2+} (–); 40 mM lysine ( ); 40 mM lysine + 300 μM Cu^{2+} ( ); 40 mM MG (–); 40 mM MG + 300 μM Cu^{2+} (–); 40 mM MG + 40 mM lysine (–) and 40 mM MG + 40 mM lysine + 300 μM Cu^{2+} (–).

Fig. 4 Agarose gel electrophoresis of native and modified human DNA. DNA samples from lane 2-5 are treated with MG-lysine (40 mM each) and Cu^{2+} (300 μM).

Lane 1: Native human DNA.
Lane 2: Modified human DNA with 3 hr. incubation.
Lane 3: Modified human DNA with 6 hr. incubation.
Lane 4: Modified human DNA with 12 hr. incubation.
Lane 5: Modified human DNA with 24 hr. incubation.

Fluorescence studies of native and MG-Lys-Cu^{2+} glycated human DNA

Generation of fluorogenic AGEs in glycated-DNA samples was measured using excitation wavelength of 370 nm (λ_{ex}) and emission wavelength of 450 nm (λ_{em}) (Table 4). This is a characteristic excitation and emission wavelength of AGEs fluorophor. Under identical conditions, native human DNA alone does not give any fluorescence. Glycation of DNA by methylglyoxal and lysine in the presence copper sulphate generated fluorescent DNA-AGEs characterized by emission maxima of 450 nm. An increase of 76.2% of fluorescence intensity was observed in glycated DNA when compared to native form (Fig. 5).

Circular dichroism of native and MG-Lys-Cu^{2+} glycated human DNA

Circular dichroism is used for elucidating conformational changes in biomacromolecules, which measures differential absorption of right and left circularly polarized light. The CD spectra of nucleic acids result primarily due to the spatial asymmetry of the constituent nucleotides. The CD profile of human DNA (37.8 μM), was recorded at a wavelength range of 220 – 400 nm which exhibited a negative peak at 243 nm, and a positive peak at 275 nm. (Fig. 6). Structural changes in DNA were evaluated by ellipticity measurements. The CD signal of modified analogue shifted from 275 to 278 nm in the direction of higher wavelength, which is indicative of structural changes in DNA. When native DNA was compared with the modified DNA, it showed an increase in ellipticity from 6.64 to 8.47 mdeg at 275 nm. This increase in ellipticity corresponds to 27.56 % structural loss in modified DNA. The structural loss in DNA after modification might be due to unstacking of bases as a result of helix destabilization.

Genotoxicity of advanced glycation end-products in human lymphocytes by single cell gel electrophoresis (comet assay)

The photographs of comets seen on treatment of lymphocytes with different combinations of MG (40 mM), lysine (40 mM) and Cu^{2+} (300 μM) are shown in Fig. 7. A comet with a tail is indicative of DNA breakage in single cell gel electrophoresis. The results clearly establish that MG in combination with lysine is capable of DNA breakage in lymphocytes as evident from the formation of distinct

TABLE 4

Fluorescence characteristics of native and modified human DNA

Parameter	Native DNA	Modified DNA
Fluorescent intensity (I_f)	13.6	44.14
Wavelength for max I_f	426	450
Stokes shift (nm)	-	24
Ratio of quantum efficiency	3.25	1

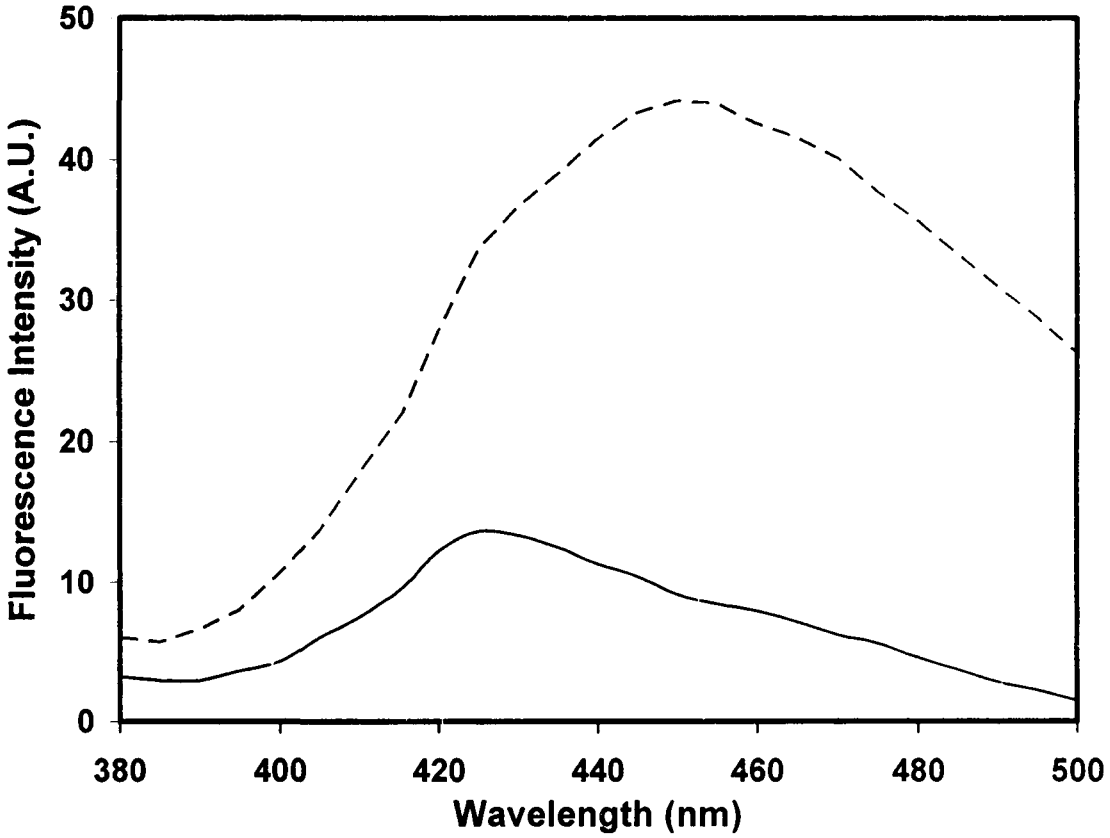


Fig. 5 Fluorescence emission spectra of native human DNA (—) and modified human DNA with 40 mM MG + 40 mM lysine + 300 μM Cu^{2+} (----).

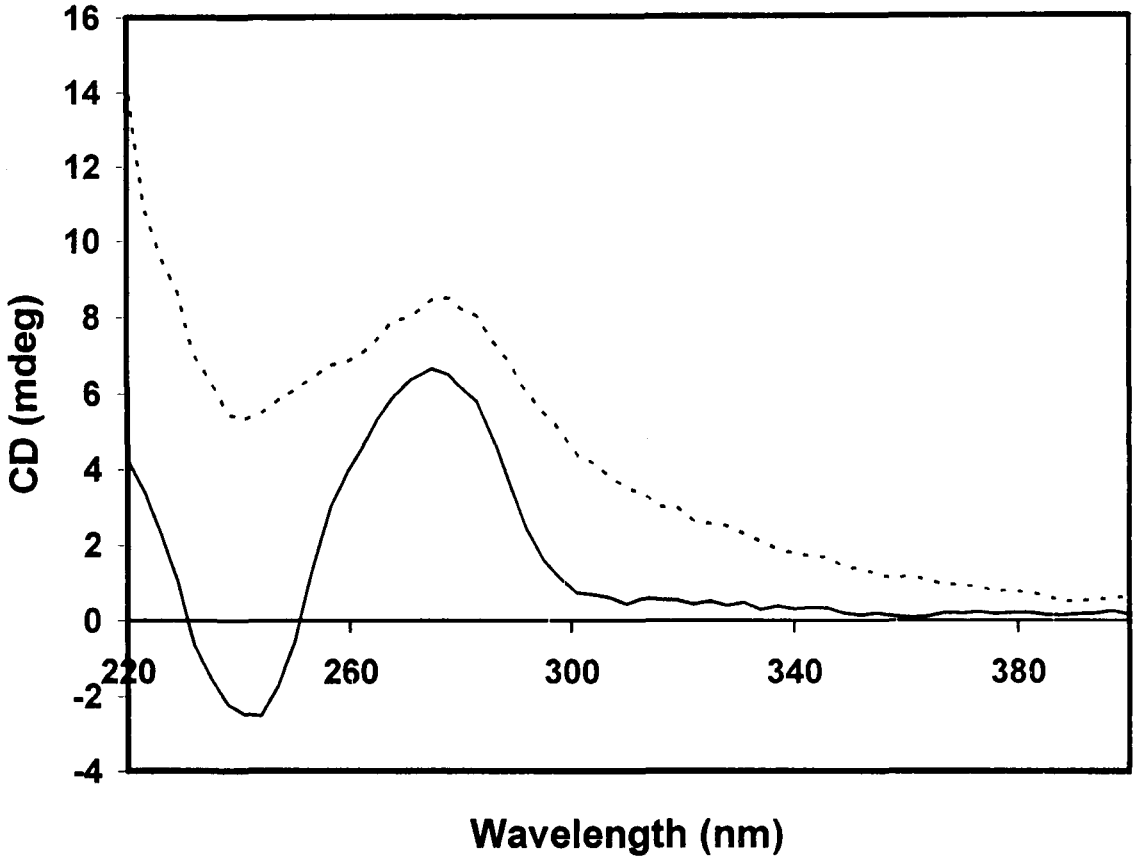


Fig. 6 Circular dichroic spectra of native human DNA (—) and modified human DNA with 40 mM MG + 40 mM lysine + 300 μM Cu^{2+} (----).

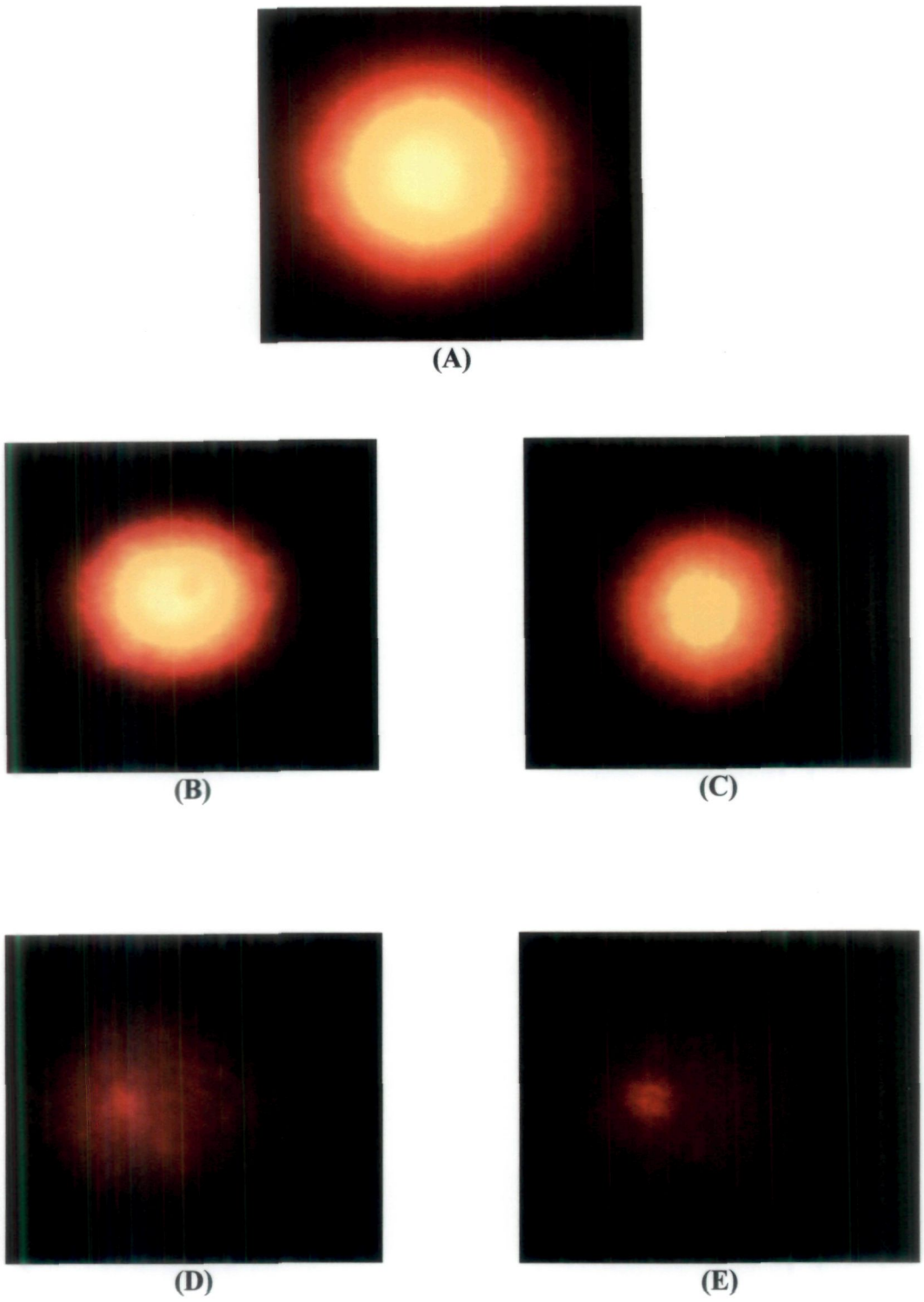


Fig. 7 Single cell gel electrophoresis of human lymphocytes showing comets after treatment with MG or lysine and Cu^{2+}, (A) untreated, (B) treated with 40 mM lysine, (C) with 40 mM MG, (D) with 40 mM MG + 40 mM lysine and (E) with 40 mM MG + 40 lysine + 300 μM Cu. All the lymphocytes were treated for 24 hour at 37 ^{o}C.

tail from the diffused head. However, in the presence of Cu^{2+} the damage of lymphocyte DNA was found to be enhanced by 36% (Fig. 7 E). This suggests that Cu^{2+} has enhanced the genotoxicity of MG+Lys system by increasing free radical ($O_2^{\cdot-}$ and $^{\cdot}OH$) generation. Furthermore, MG and lysine alone did not cause a significant damage to the lymphocyte DNA.

The DNA damage parameters i.e. olive tail moment (OTM), mean percentage of tail DNA and tail length were also measured (Figs. 8-10) and found to be significantly increased in MG-Lys-Cu^{2+} system as compared to MG-Lys system. A marked increase in OTM (316%) and mean percentage of tail DNA (246%) was observed in MG-Lys-Cu^{2+} treated lymphocytic cells when compared to control (untreated lymphocytes). Furthermore, there was a pronounced increase in tail length, which was found to be 92% as compared to control.

Nuclease S1 digestibility of native and MG-Lys-Cu^{2+} glycated human DNA

Native and modified DNA were digested with nuclease S1 (20 units/mg DNA) for 30 minutes and electrophoresed on 0.8% agarose to visualize the generation of single strand breaks. The controls were native and modified DNA samples, untreated with nuclease S1. Modified DNA showed decrease in fluorescence intensity following nuclease S1 digestion. On the other hand nuclease S1 treated and untreated native DNA showed almost identical electrophoretic migration pattern and fluorescence intensity (Fig. 11) .These observations clearly demonstrate that sufficient distortions (formation of single strand breaks) are caused in the helical structure of DNA by MG-Lys-Cu^{2+} treatment, rendering it susceptible to digestion by single strand specific nuclease S1.

Thermal denaturation of native and MG-Lys-Cu^{2+} glycated human DNA

Thermally induced transitions were measured spectrophotometrically at 260 nm by heating nucleic acid samples at a rate of 1 $^{\circ}C$/ minute. Melting profile of modified and unmodified human DNA were analysed in the temperature range of 30 $^{\circ}C$ to 95 $^{\circ}C$. Increase in absorbance at 260 nm was taken as a measure of helix denaturation. The process was characterized by determining the percent DNA in

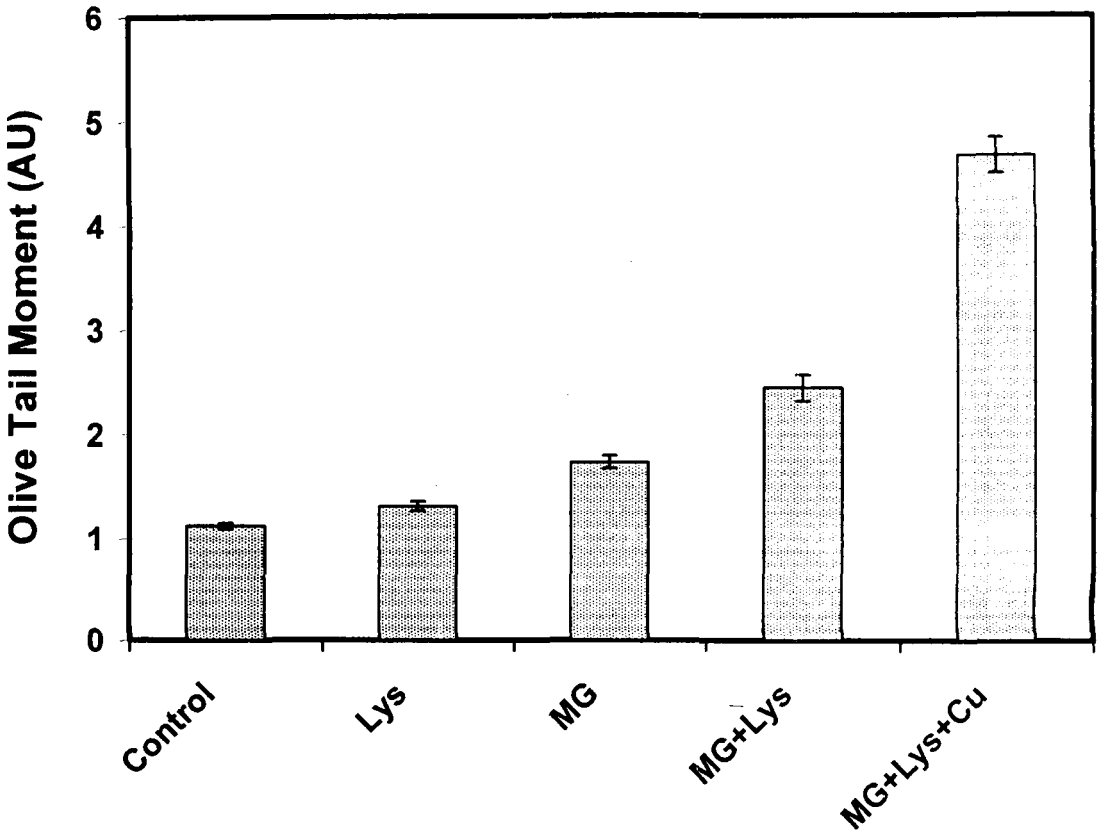


Fig. 8 Effect of lysine (40 mM), MG (40 mM), MG + lysine (40 mM each), and MG + lysine (40 mM each) + Cu^{2+} (300 μM) on olive tail moment in lymphocytic DNA.

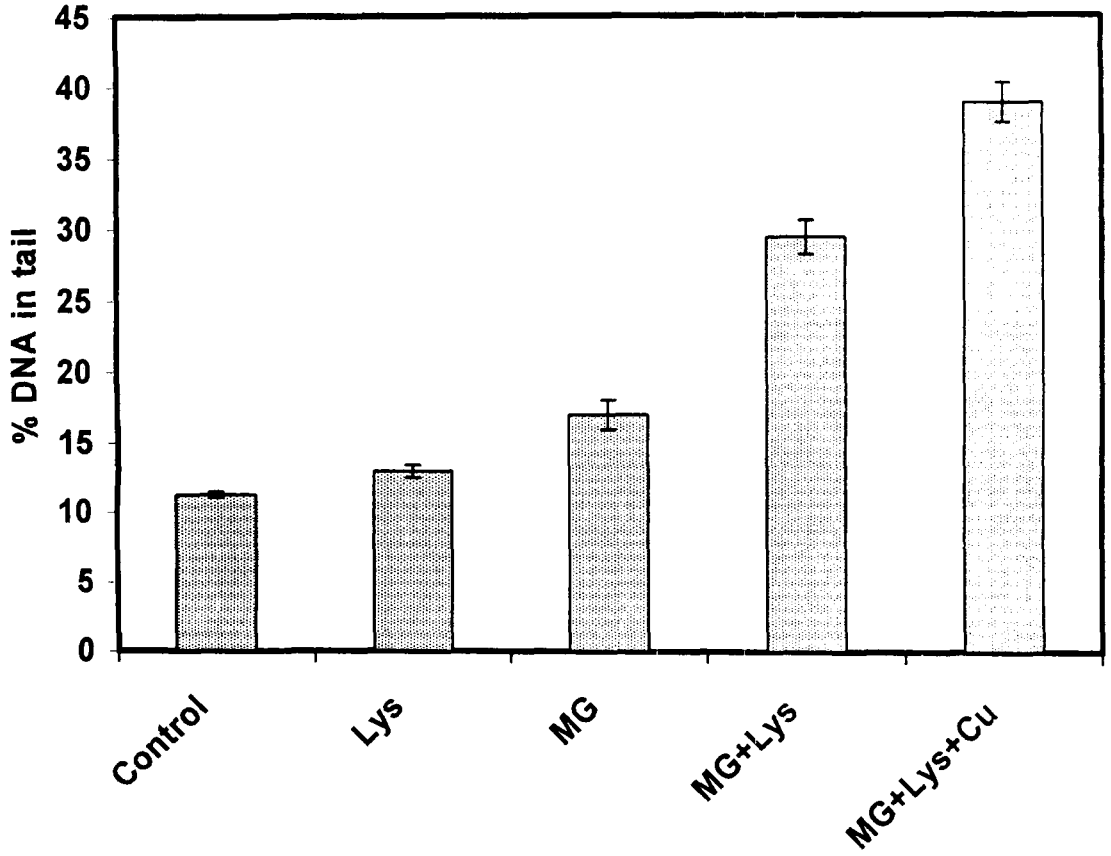


Fig. 9 DNA damage (% DNA in tail) in the comet assay in peripheral lymphocytes after treatment with lysine (40 mM), MG (40 mM), MG + lysine (40 mM each) and MG + lysine (40 mM each) + Cu^{2+} (300 μM).

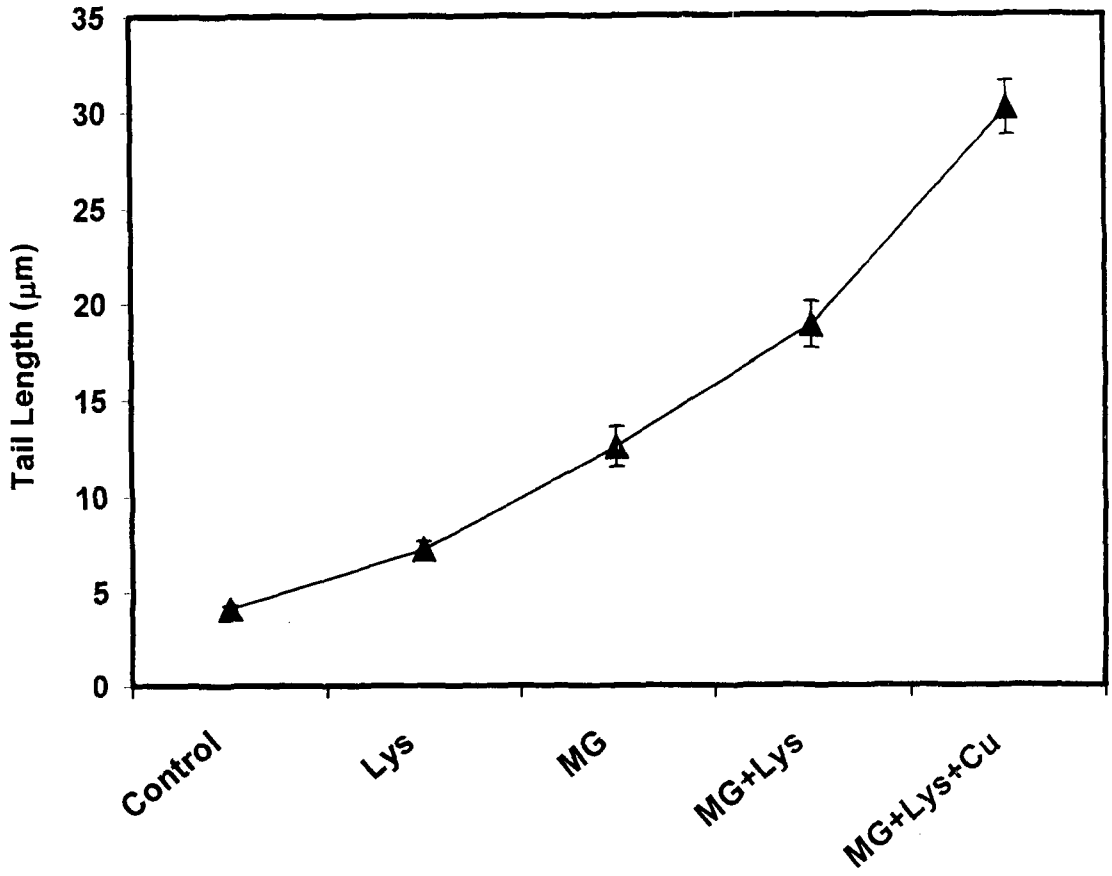


Fig. 10 DNA damage (Tail length, μm) in the comet assay in peripheral lymphocytes after treatment with 40 mM of lysine; 40 mM of MG; MG + lysine (40 mM each) and MG + lysine (40 mM each) + Cu^{2+} (300 μM).

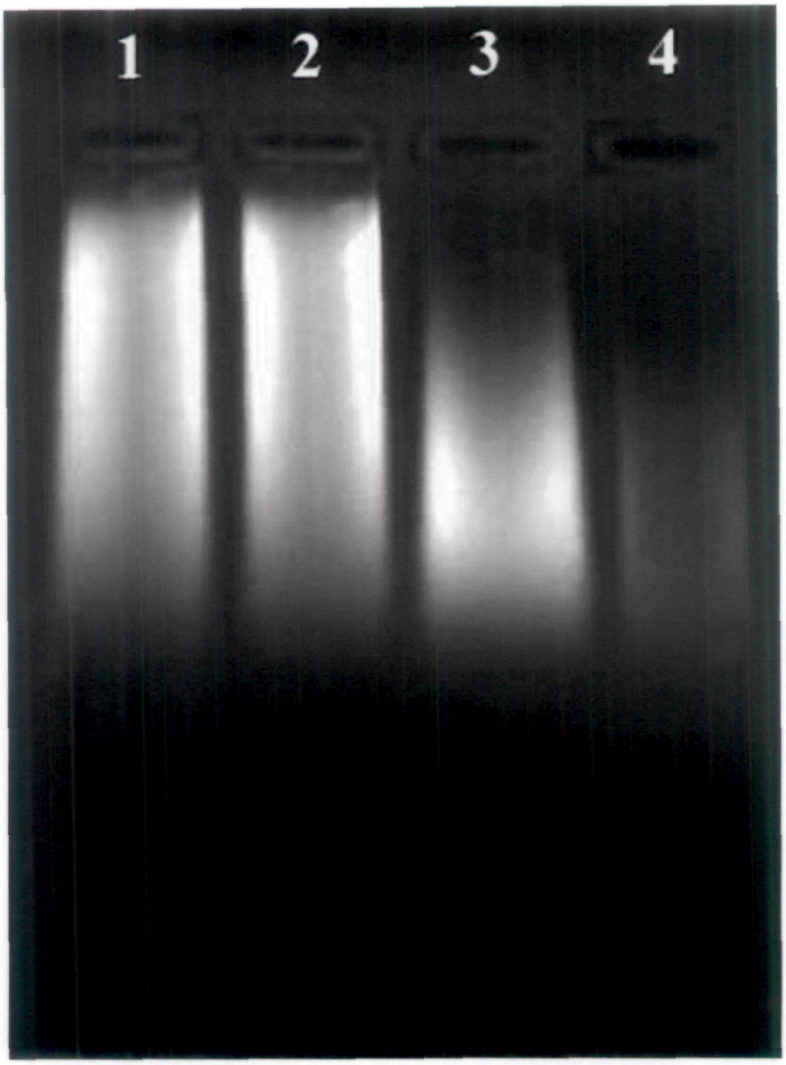


Fig. 11 Nuclease S1 digestibility of native and modified DNA. Lane 1 contains native DNA, while Lane 2 contains native DNA treated with nuclease S1 for 30 min; Lane 3 contains modified DNA and Lane 4 contains modified DNA treated with nuclease S1 for 30 min. Electrophoresis was carried out on 0.8% agarose gel for 2 hr at 30 mA.

denatured state as a function of temperature. The melting temperature, T_m, of native DNA was found to be 86 ^oC, while in case of modified DNA it was recorded at 76 ^oC, which shows a significant decrease in the T_m value of modified DNA as compared to its unmodified native form (Fig. 12). A decrease of 10 ^oC in the T_m value in case of modified human DNA point towards alterations in the DNA molecule as a result of modification that may be unstabilizing the helix. Generation of strand breaks and base modifications cause altered hydrogen bonding between base pairs, resulting in thermal susceptibility of the DNA molecule. Moreover, the melting curve for modified DNA indicated a progressive and heterogeneous local melting with the increase in temperature as compared to more homogenous global melting in the control. Early onset of melting in the case of modified DNA is a definite indication of structural instability consequent to modification. The thermal denaturation characteristics of native and modified DNA are listed in Table 5.

Quenching studies

The modification of human DNA in the present study is a result of production of $^{\bullet}OH$ and $O_2^{\bullet -}$. Its *in vitro* generation was confirmed by the use of different quenchers of various radicals. Quenchers like SOD, mannitol, EDTA and catalase were exploited to study their quenching effect on MG-Lys-Cu^{2+} modified human DNA. As evident from the figure 13, the hydroxyl radical ($^{\bullet}OH$) trapping agent (mannitol) and superoxide radical ($O_2^{\bullet -}$) trapping agent (SOD) strongly inhibited (82.6% and 78.3%, respectively) the modification by MG-Lys-Cu^{2+} system, thereby indicating the involvement of these radicals. Whereas EDTA showed marked inhibition of 69%. Moreover, uric acid, an antioxidant inhibited DNA modification to the extent of 46%.

Quantitation of hydroxyl radical in MG-Lys-Cu^{2+} system

The generation of hydroxyl radicals in the MG-Lys-Cu^{2+} system was measured with thiobarbituric acid-reactive substance (TBARS). The incubation of 2-deoxy-D-ribose with lysine and MG produced 13 nmol TBARS ml^{-1} (Fig. 14). However, reaction of MG with lysine in the presence of Cu^{2+} enhanced it to 24 nmol TBARS ml^{-1}. Radical scavengers like mannitol, catalase and a metal ion chelator, desferrioxamine significantly inhibited the production of TBARS (Fig. 15). TBARS

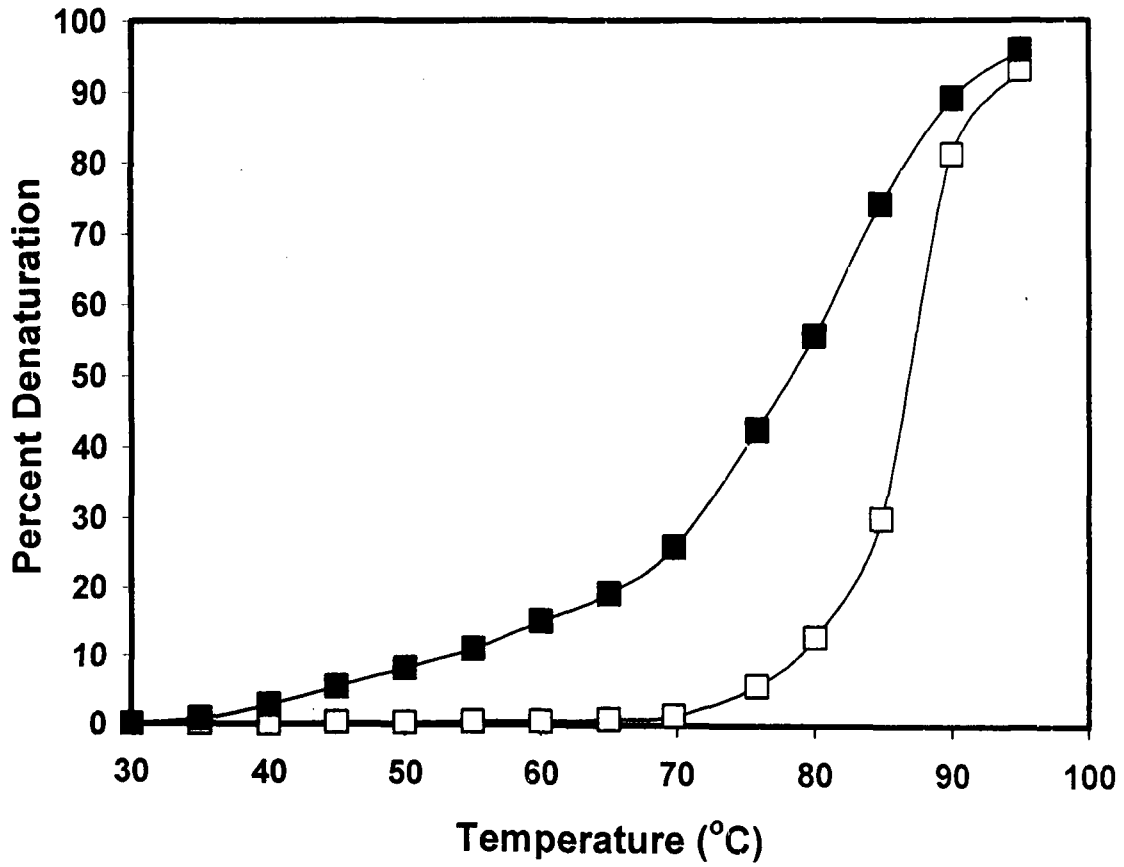


Fig. 12 Thermal melting profile of native human DNA (□) and modified human DNA (■).

TABLE 5

Ultraviolet and thermal denaturation characteristics of native and modified human DNA

Parameter	Native DNA	Modified DNA
Absorbance ratio (A_{260}/ A_{280})	1.76	1.4
Hyperchromicity at 95 °(%)	36.5	24
Melting temperature (Tm), °C	86	76
Onset of duplex melting, °C	72	36

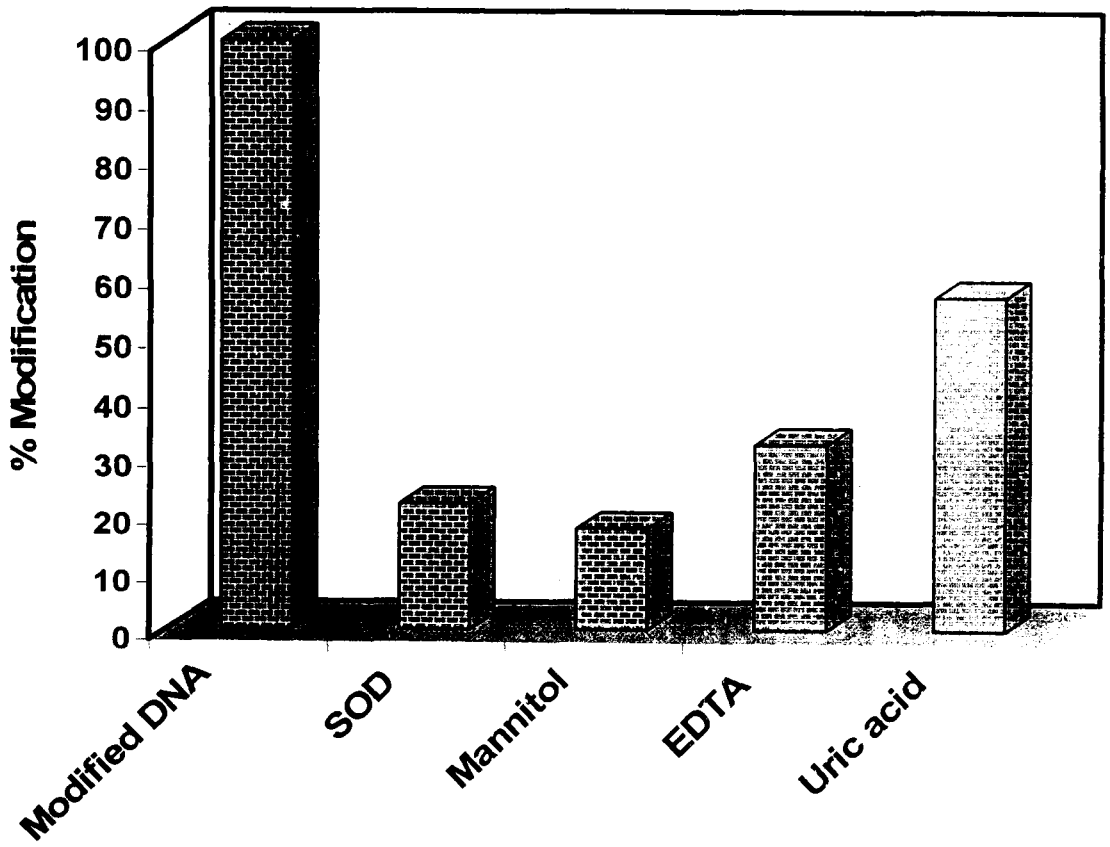


Fig. 13 Effect of various scavengers of free radical (mannitol, SOD), metal ion chelator (EDTA) and antioxidant (uric acid) on human DNA modification induced by MG-Lys-Cu^{2+} system.

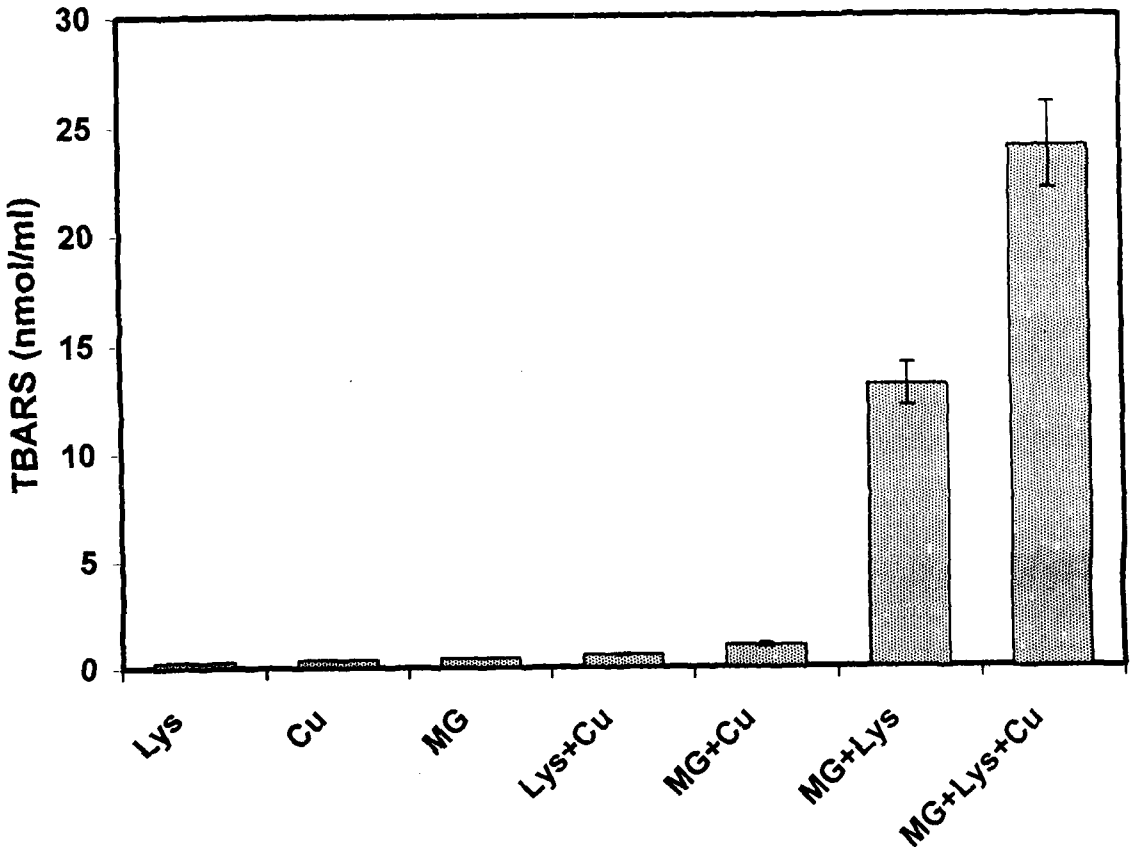


Fig. 14 Generation of hydroxyl radicals during the glycation reaction of lysine with MG in the presence of Cu^{2+}. The reaction mixtures contained 100 mM 2-deoxy-D-ribose in 10 mM phosphate buffer at pH 7.4 and the following: 40mM MG, 40mM lysine, 300μM Cu^{2+} with their different controls as depicted in diagram. Data represent the means ± S.D. (n=4).

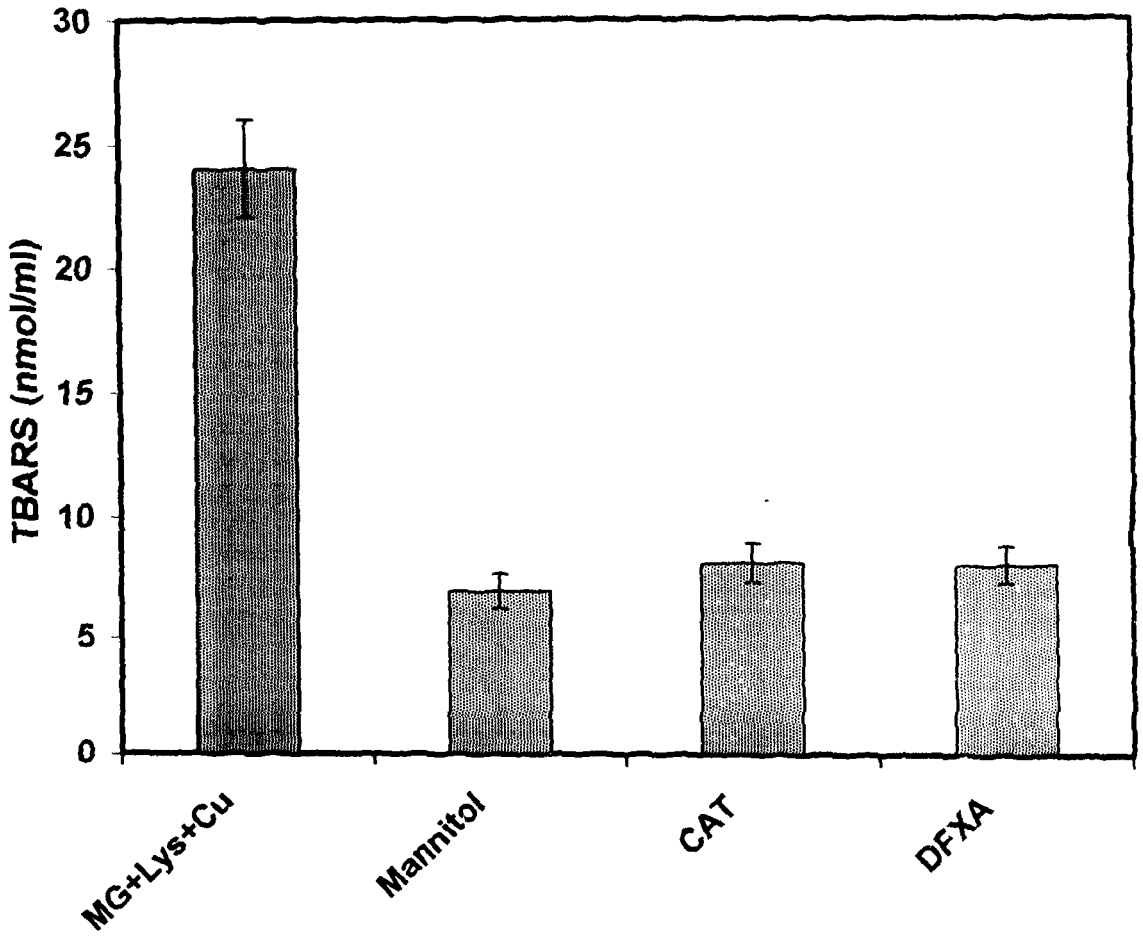


Fig. 15 The reaction mixtures contained 100 mM 2-deoxy-D-ribose along with 40 mM each of MG and lysine and 300μM Cu^{2+} in the presence of radical scavenger, mannitol (50 mM), catalase (CAT) (2mg/ml) and a metal ion chelator, Desferrioxamine (DFXA) (10mM). Data represent the means ± S.D. (n=4).

generation was inhibited upto 71.7% when mannitol was used as scavenger. However, both catalase and DFXA inhibited TBARS equally, which was found to be almost 67%. The result suggests that the MG-mediated hydroxyl radical generation may be caused by traces of transition metals. Furthermore, it is also being suggested that the redox reactions of copper may facilitate the generation of hydroxyl radical by reaction of MG and lysine.

Quantitation of superoxide anion in MG-Lys system

Superoxide generation was quantitated by cytochrome c reduction experiment. During incubation of MG with lysine, the formation of superoxide anion was gradually increased in time dependent manner. The incubation of MG (40 mM) with lysine (40 mM) produced 28.2 nmol $O_2^{\cdot-}$ ml^{-1} h^{-1} compared to 2.46 nmol $O_2^{\cdot-}$ ml^{-1} h^{-1} with MG (40 mM) alone and 1.96 nmol $O_2^{\cdot-}$ ml^{-1} h^{-1} with lysine (40 mM) alone (Fig. 16). Superoxide dismutase (SOD) inhibited the superoxide radical generation. As evident from figure 17, on increasing concentration of SOD, the superoxide generation was gradually decreased.

Anti-glycation study

Anti-glycating agent D-penicillamine (1 mM) and pyridoxal phosphate (PLP) (10 mM) showed remarkable inhibition of 55% and 73% respectively in DNA modification as analyzed at 260 nm. However, at 330 nm D-penicillamine and PLP showed still higher inhibition of 69% and 85% respectively (Fig. 18). The enhanced inhibition at 330 nm is due to the formation of AGEs at this wavelength range (330-360 nm) (Schmitt *et al.*, 2005). This clearly indicates the formation of AGEs in the MG-Lys-Cu^{2+} modified human DNA.

Synthesis and characterization of N^2-(1-Carboxyethyl)-2-deoxyguanosine (CEdG)

Synthesis of the standard, CEdG was performed as described by (Seidel and Pischetsrieder, 1998) with slight modifications. After final preparation, CEdG was isolated by preparative HPLC using 50 mM ammonium acetate buffer solution and methanol as eluents. The elution of CEdG was obtained at a retention time of 14.399

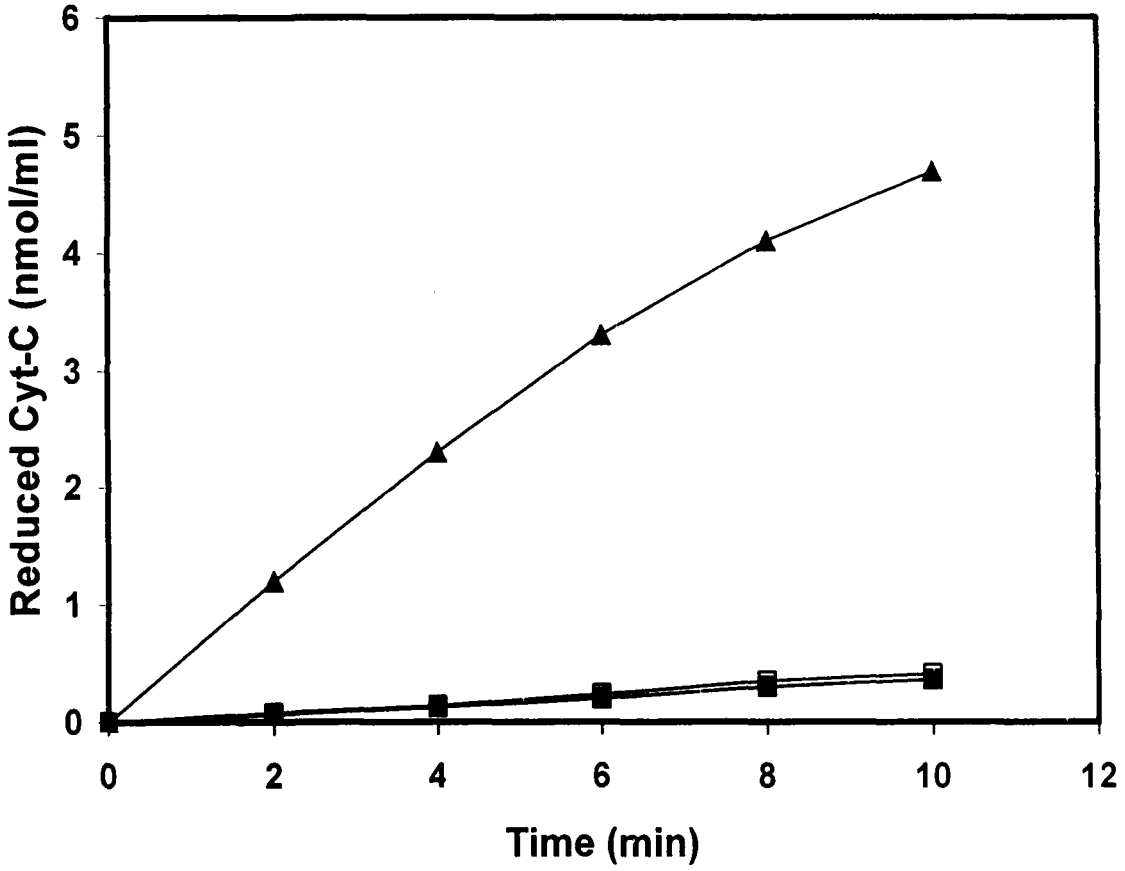


Fig. 16 Generation of the superoxide anion in the glycation reaction of lysine with MG. Reduction of cytochrome c was measured by increasing concentrations of reaction products. The reaction mixture contained 10 μM cytochrome c in 10 mM phosphate buffer at pH 7.4 and the following: 40 mM MG (□); 40 mM lysine (■); 40 mM MG and lysine (▲). The absorbance changes were monitored at 550 nm for 10 min at room temperature.

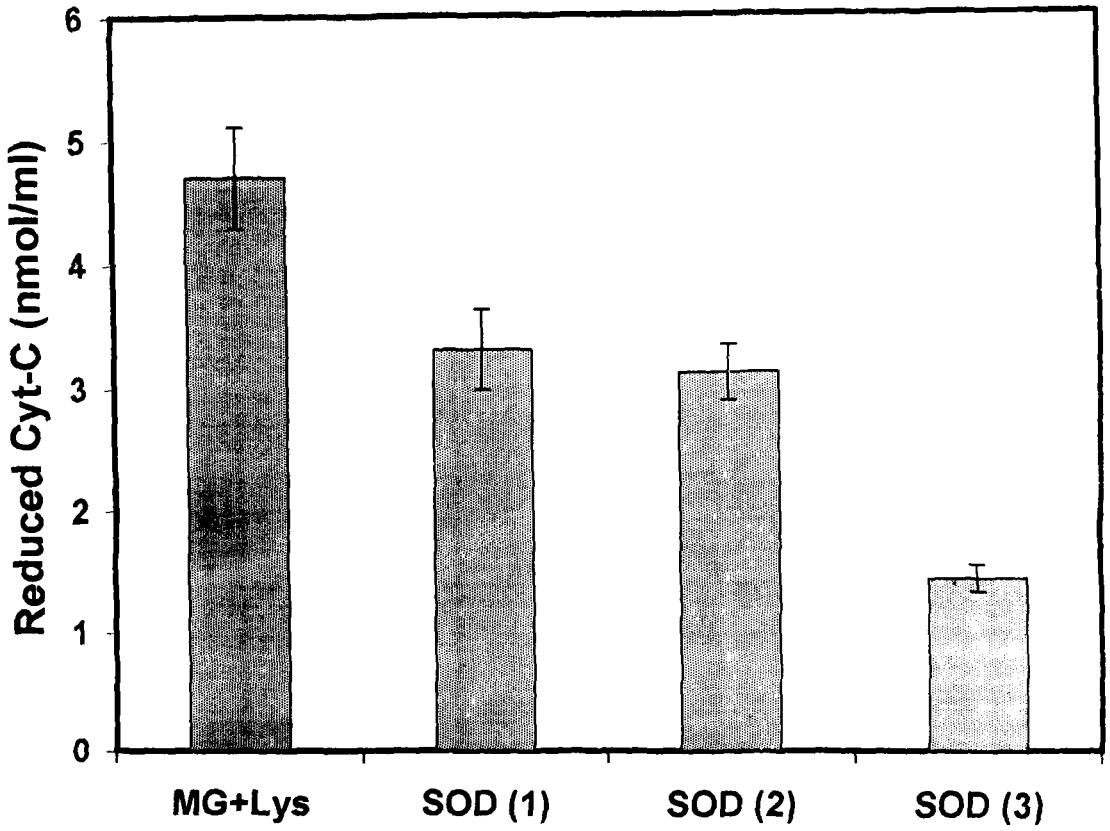


Fig. 17 Superoxide formation during the glycation reaction of lysine by MG. MG (40 mM) and lysine (40 mM) was reacted in presence of various concentrations of superoxide dismutase, 50 units (1), 100 units (2) and 500 units (3).

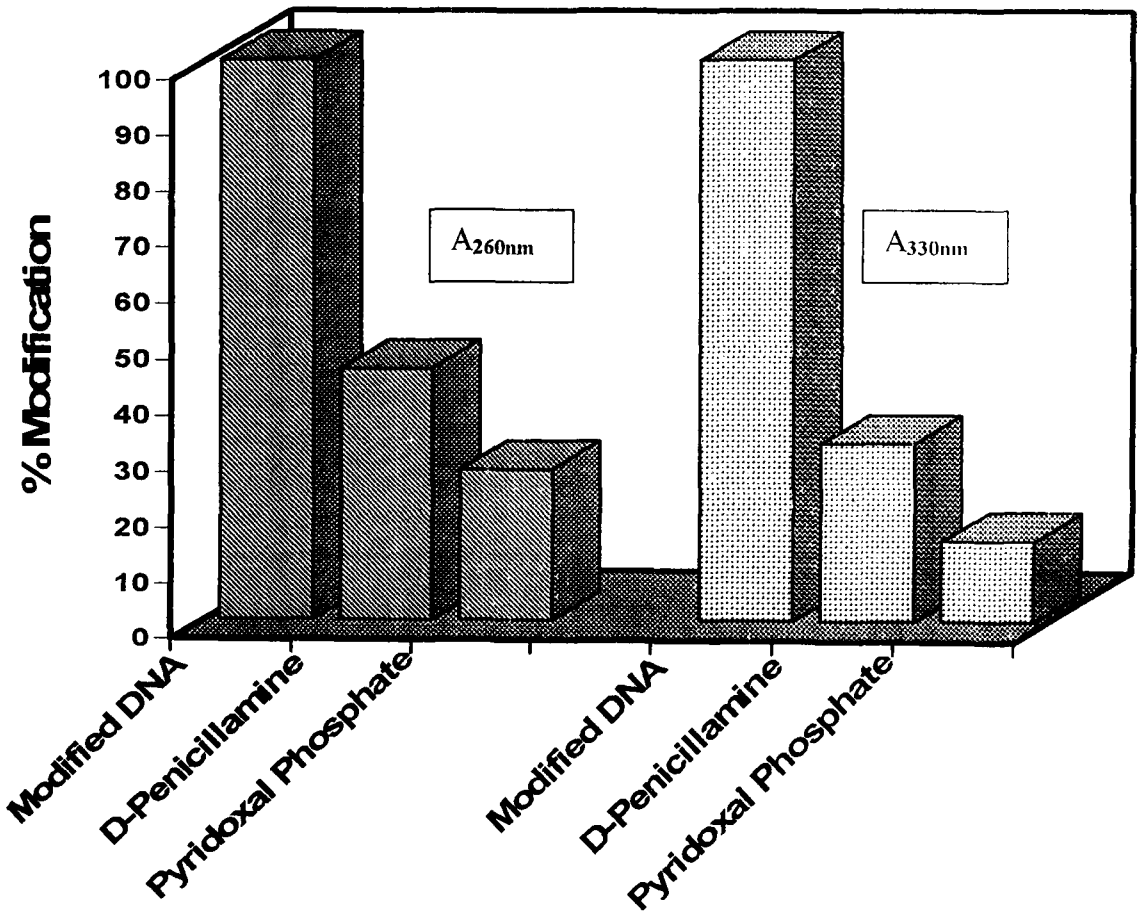


Fig. 18 Effect of antiglycating agent D-Penicillamine (1 mM) and pyridoxal phosphate (10 mM) on the modification of DNA induced by MG-Lys-Cu^{2+} system. Percent DNA modification was calculated at 260 nm and 330 nm after 24 hr incubation.

min when UV detector was used for the experiment. However, deoxyguanosine (dG) gave elution at a retention time of 9.1 min (Fig. 19).

Nuclear magnetic resonance of CEdG

For structural assignment of the compound, the CEdG peak was isolated by HPLC and then subjected to ^{1}H NMR analysis. Resonance signals can be identified and characterized in ^{1}H NMR spectrum of CEdG (Fig. 20), recorded in DMSO-d6. ^{1}H NMR (400MHz, DMSO-d6, 20 °C) assignment for CEdG: δ 10.6 (s,1H, N1-H), δ 7.93 (s, 1H, C8-H), δ 6.76 (d, 1H, C2-NH), δ 6.12 (dd, 1H, C1′-H), δ 5.3 (d, 1H, C3′-OH), δ 4.89 (s, 1H, C5′-OH), δ 4.36 (m, 1H, C2-NH-CH), δ 4.18 (q, 1H, C2-NH-CH), δ 3.81 (m, 1H,C3′-H), δ 3.5 (ddd, 2H, C5′-H_2), δ 2.64 (ddd, 1H, C2′-H), δ 1.39 (d, 3H, C2-NH-CH-CH_3).

The ^{1}H NMR analysis indicated a –CHX-CH_3 group bound to the purine (guanine) ring. The chemical shifts (δ), 4.18 ppm (q, 1H, C2-NH-CH) and 1.39 ppm (d, 3H, C2-NH-CH-CH_3) respectively, is attributed to the carboxyethyl group of the standard carboxyethyl-deoxyguanosine (CEdG).

Characterization of native and MG-Lys-Cu^{2+} glycated human DNA by HPLC

Figures 21 and 22 show the representative HPLC chromatograms of acid hydrolysed samples of native and modified human DNA respectively. Well defined peaks at retention time 4.467 min, 7.332 min, 8.727 min were observed in native DNA. However, in the case of modified DNA these peaks shifted to 6.748 min, 8.455 min and 12.399 min respectively, suggesting considerable change in the DNA bases. The extra peak at a retention time of 14.249 min in the acid hydrolysate of modified DNA is characteristic of N^2-(1-Carboxyethyl)-2-deoxyguanosine (CEdG) adduct. This is in conformity with the standard CEdG results wherein also when deoxyguanosine was exposed to dihydroxyacetone, a distinct peak at retention time of 14.399 min was observed (Fig. 19). The CEdG adduct is a marker of glucose and MG induced DNA glycation.

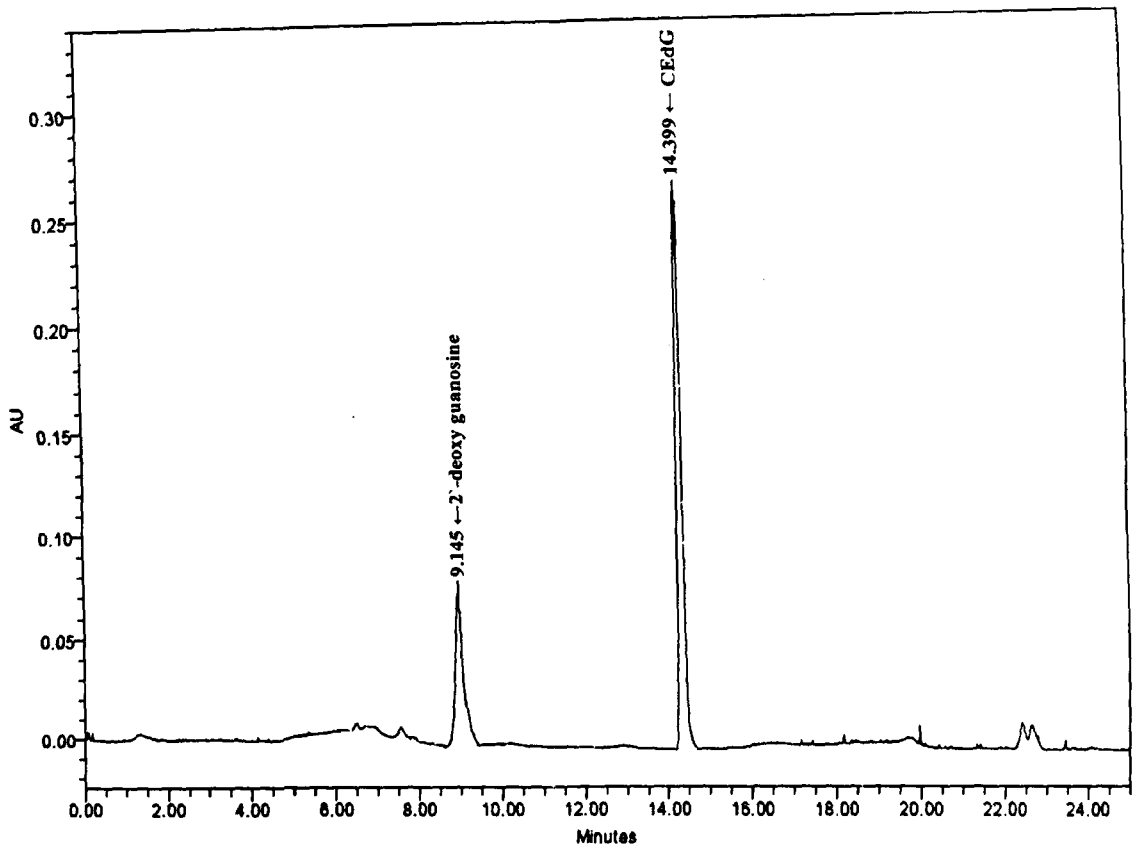


Fig. 19 Representative HPLC chromatogram of the reaction of 2'- deoxyguanosine with dihydroxyacetone.

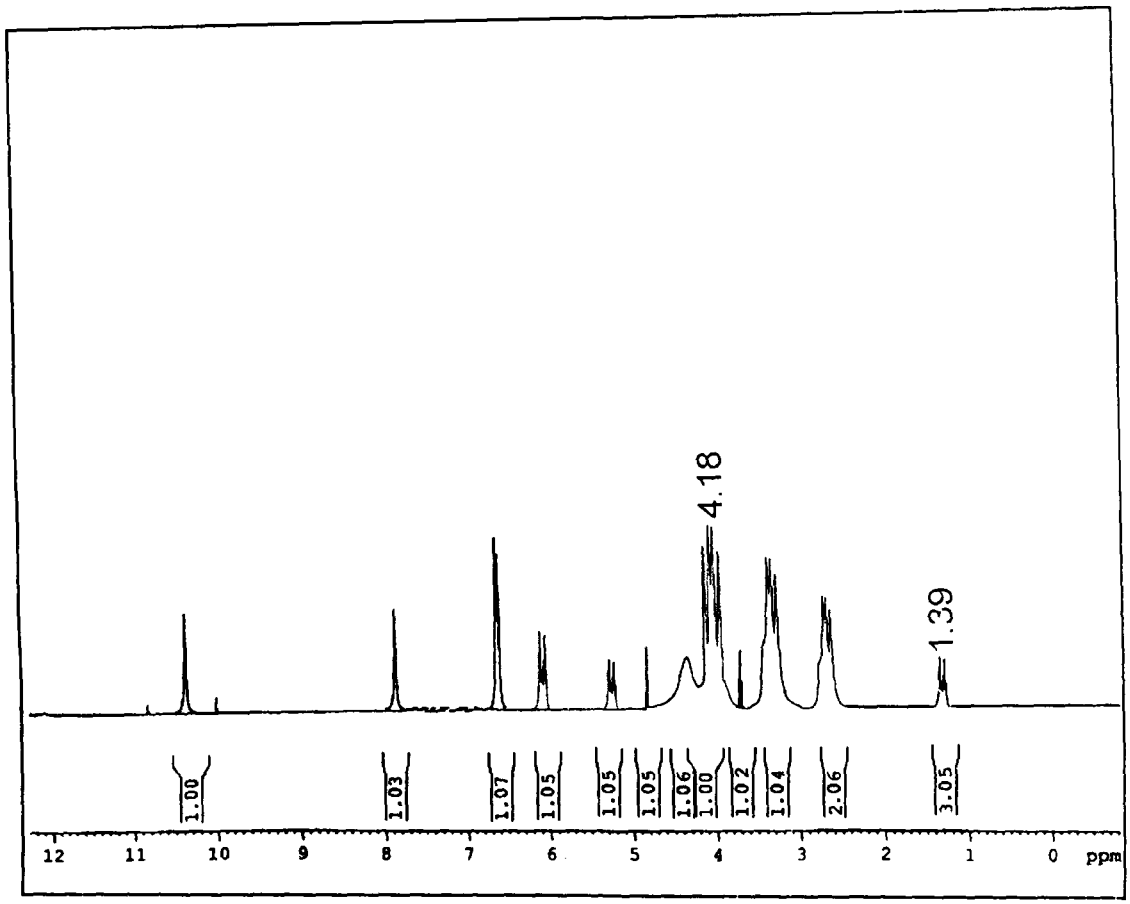


Fig. 20 ^{1}H NMR spectra of the standard CEdG.

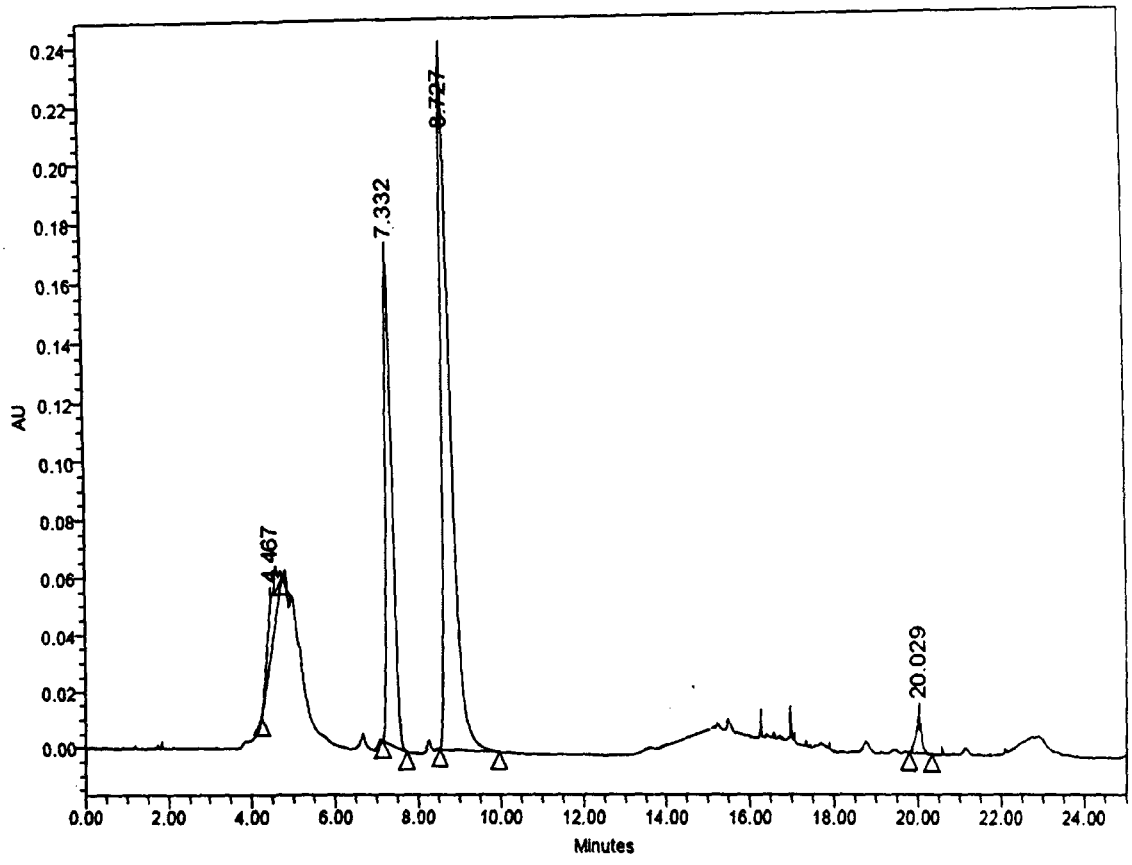


Fig. 21 Representative HPLC chromatogram of acid hydrolysate of native human DNA.

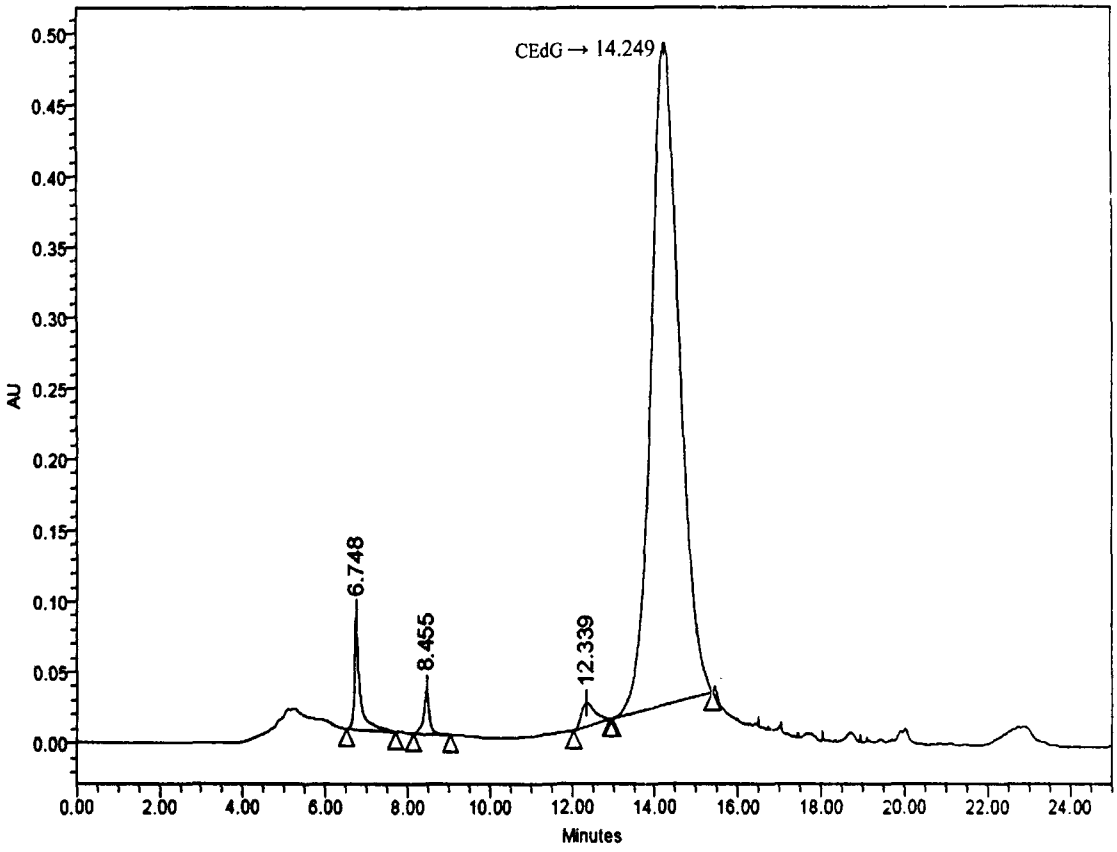


Fig. 22 Representative HPLC chromatogram of acid hydrolysate of modified human DNA.

Detection of N^2-(1-Carboxyethyl)-2-deoxyguanosine (CEdG) formed in modified human DNA by LC-MS

The UV spectrum of the adduct showed the maxima at 254 nm same as that for unmodified deoxy-guanosine, indicating that it is the modification of same nucleoside. The standard (CEdG) and modified DNA samples were then analyzed by mass spectrometer for full scan in negative mode, giving evidence for the adduct formed $[M- H]^-$ at m/z values of 338 (Figs. 23-25). In addition, the observed masses corresponded with the calculated masses for single charged ions $[M- 1]^-$ of the nucleoside plus 1 equivalent of MG (m/z 338 for dGuo). Whereas, we did not find peak at m/z value of 338 for native DNA.

Characterization of DNA-AGEs by electrospray ionization mass spectrometry (ESI-MS)

In an attempt to confirm the formation of Schiff base and amadori product in glycated DNA, mass spectrometry was used to analyze the hydrolysed glycated human DNA. Figures 26-27 show the respective mass-spectral profiles of hydrolyzed native and MG-Lys-Cu^{2+} glycated human DNA. The ion at m/z 341 is consistent with a $[Schiff\ base+H]^+$ molecule resulting from the condensation reaction of dG (Mr 285.26) with methylglyoxal (Mr 70.06) in a dehydration reaction involving the loss of a water molecule. The ion at m/z 679 is consistent with the formation of a $[Schiff\ base+H]^+$ dimer product. The ion at 268 is speculated to result from the loss of a hydroxyl group from dG. The ion with m/z 385 is assumed to be fragment formed by the degradation of methylglyoxal reacting with Schiff base product, or its enaminol or Amadori intermediates.

Immunogenicity of MG-Lys-Cu^{2+} modified human DNA

The antigenicity of MG-Lys-Cu^{2+} modified human DNA was evaluated by inducing antibodies in female rabbits. Antigenic specificity of the induced antibodies was assayed by direct binding and competition ELISA. The binding of these antibodies to the immunogen and native human DNA was further checked by gel retardation assay.

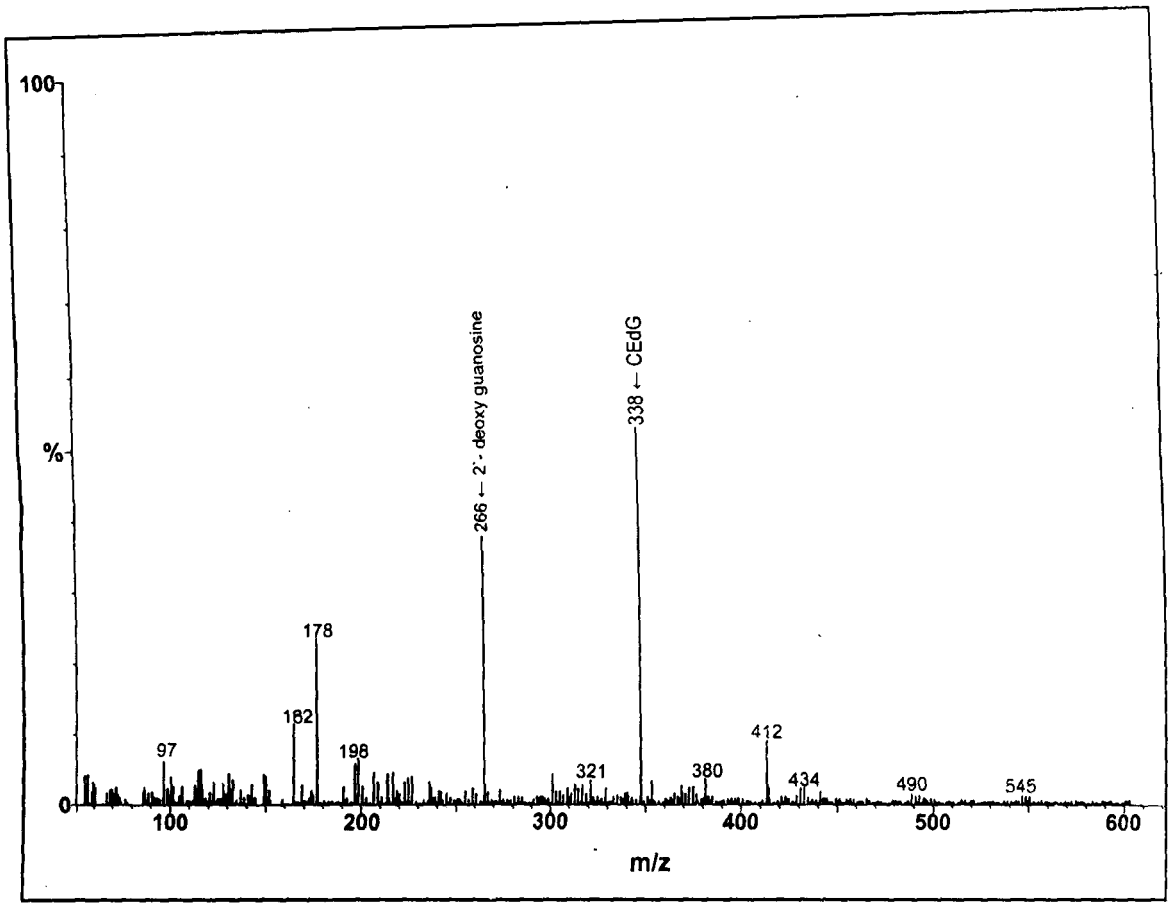


Fig. 23 Full scan LC-MS spectral analysis of synthesized CEdG standard.

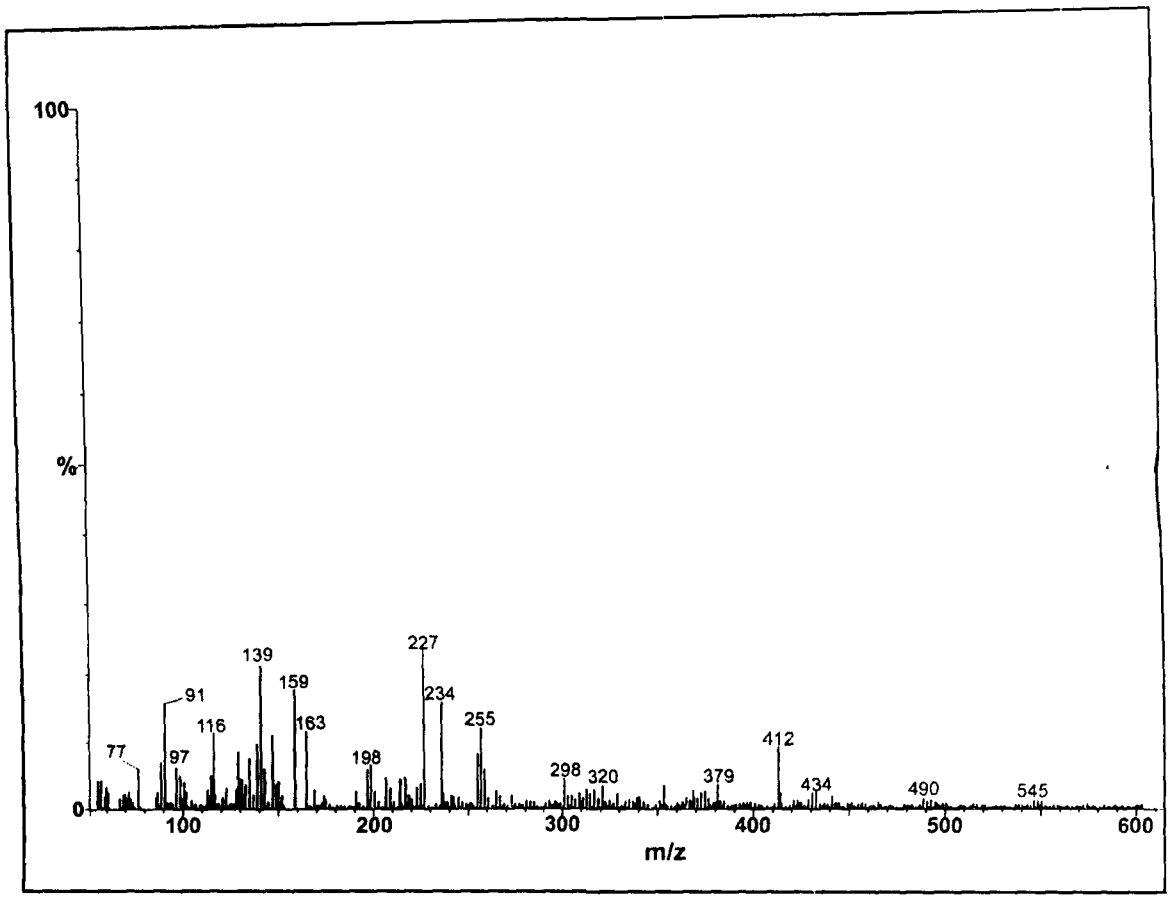


Fig. 24 Full scan LC-MS spectral analysis of hydrolyzed native human DNA (1mg/ml).

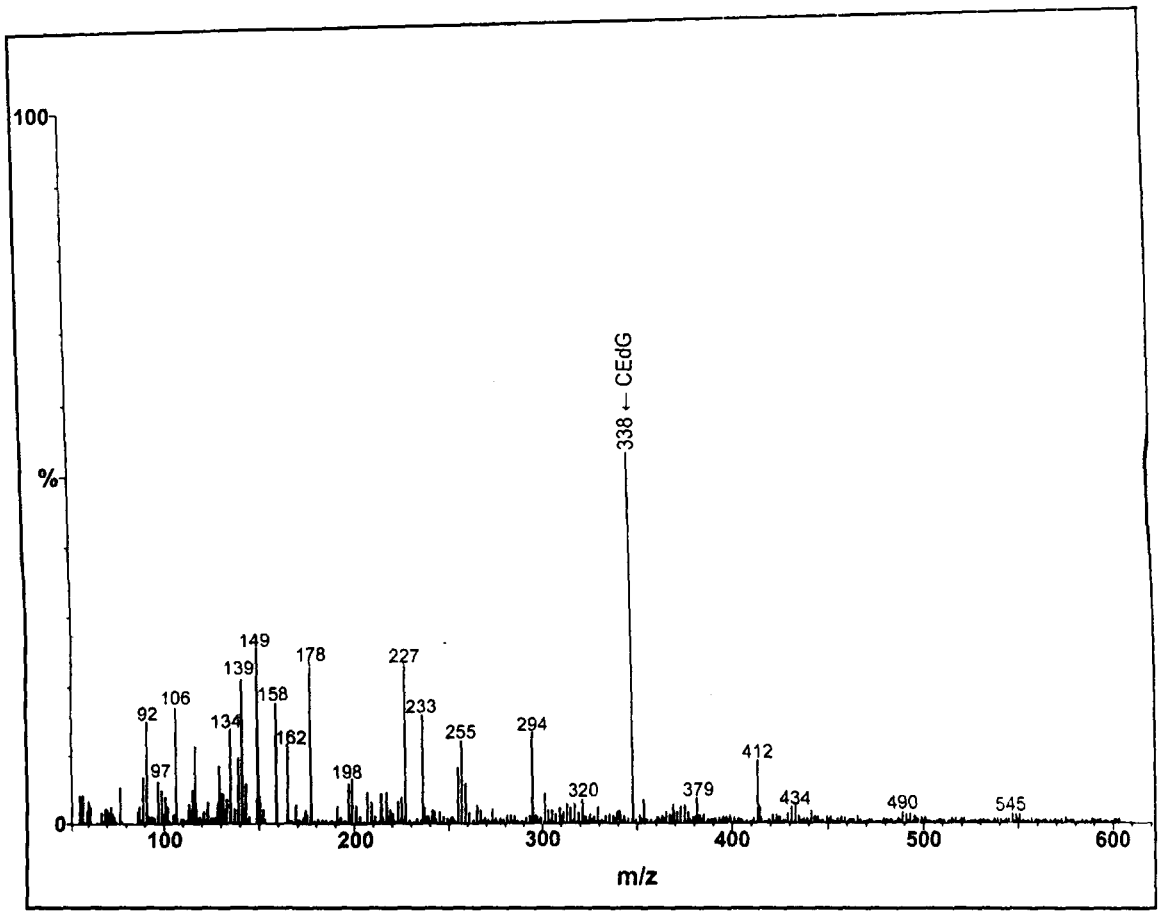


Fig. 25 Full scan LC-MS spectral analysis of hydrolyzed modified human DNA (1mg/ml).

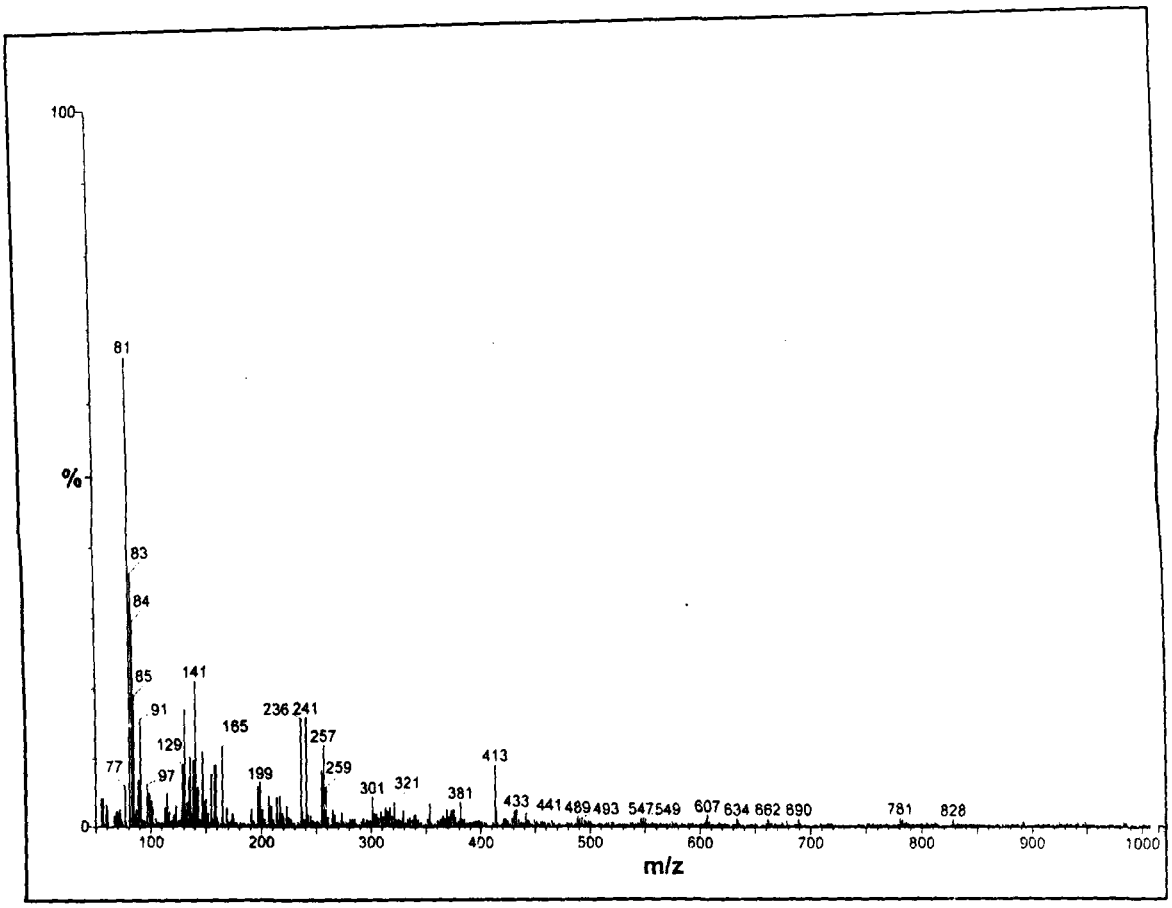


Fig. 26 Full scan ESI-MS spectral analysis of native human DNA (1mg/ml) of HPLC resolved products.

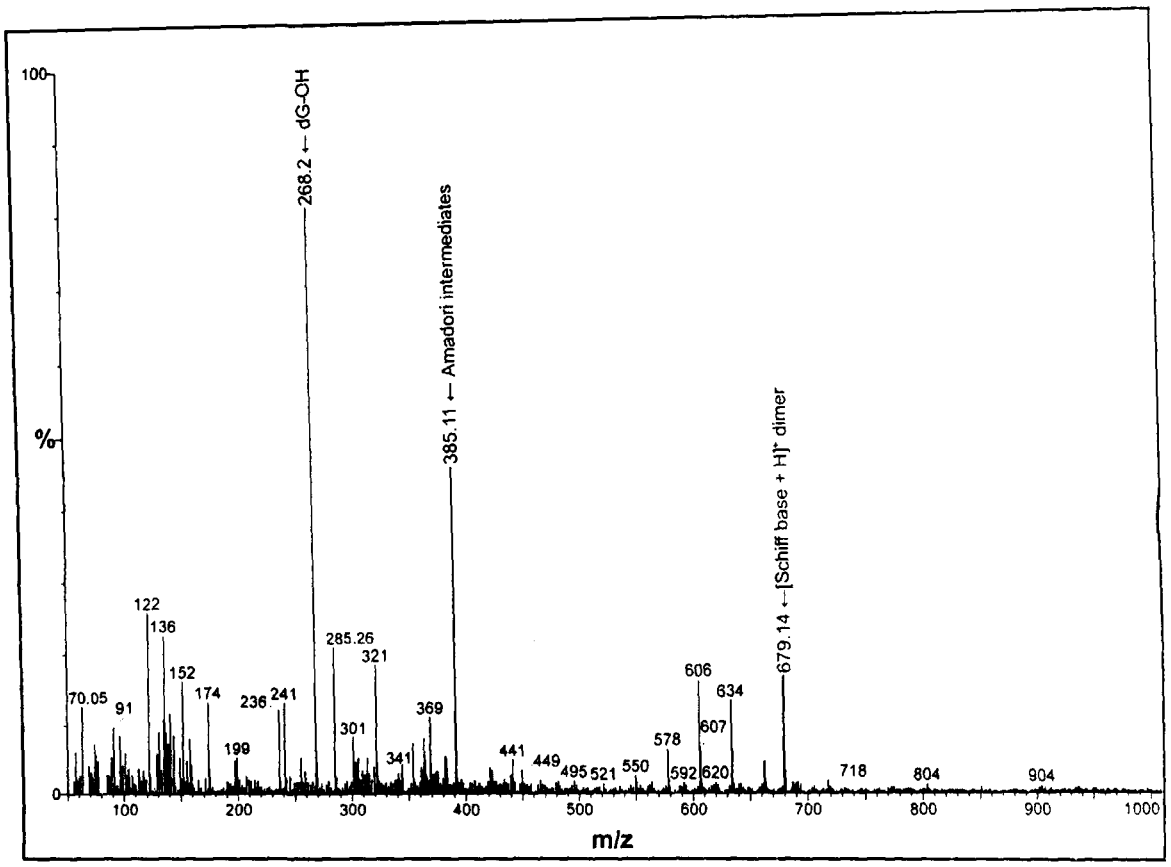


Fig. 27 Full scan ESI-MS spectral analysis of modified human DNA (1mg/ml) of HPLC resolved glycated products.

Antibodies against MG-Lys-Cu^{2+} modified human DNA

Antiserum obtained from immunized animal was subjected to direct binding immunoassay on immunogen coated polystyrene microtitre plates. The antiserum showed a high titre (> 1: 12800) in direct binding ELISA while preimmune serum, used as control, showed feeble binding on immunogen coated plates (Fig. 28). The specificity of induced anti-MG-Lys-Cu^{2+} modified human DNA antibodies was evaluated by competitive inhibition ELISA. A maximum of 62.2% inhibition in antibody binding was observed at 20 μg/ml of the immunogen (Fig. 29). The concentration for 50% inhibition was 10.1 μg/ml. While MG-Lys-Cu^{2+} modification of human DNA rendered it highly immunogenic, the native counterpart did not produce any appreciable immune response in experimental animals (Fig. 30 & 31 respectively).

Purification and characterization of serum IgG

Immunoglobulin G was isolated from preimmune and immune rabbit serum by affinity chromatography on Protein A-agarose column (Fig. 32). The purity of IgG was ascertained by SDS-polyacrylamide gel electrophoresis in absence of a reducing agent. Appearance of a single band in SDS-PAGE under non reducing condition (Fig.32 inset) establishes the purity of IgG. Direct binding ELISA of the purified anti-MG-Lys-Cu^{2+}-DNA IgG showed strong binding towards its immunogen (Fig. 33). However, the preimmune IgG showed negligible binding. The specificity of anti-MG-Lys-Cu^{2+} modified human DNA IgG was evaluated by competition ELISA, wherein 88.5% inhibition in the antibody binding was achieved by the MG-Lys-Cu^{2+} modified human DNA, while native DNA could cause only 35.2% inhibition in antibody binding (Fig. 34). Fifty percent inhibition for MG-Lys-Cu^{2+} modified human DNA binding was observed at an inhibitor concentration of 2.8 μg/ml.

Band shift assay

Antigen-antibody interaction was further evaluated by band shift assay. Constant amount of human DNA or its MG-Lys-Cu^{2+} modified form was incubated with varying amount of anti-MG-Lys-Cu^{2+}-DNA IgG for 2 hr at 37 ^{0}C and overnight at 4 ^{0}C. The resulting immune complex was then electrophoresed on 0.8% agarose

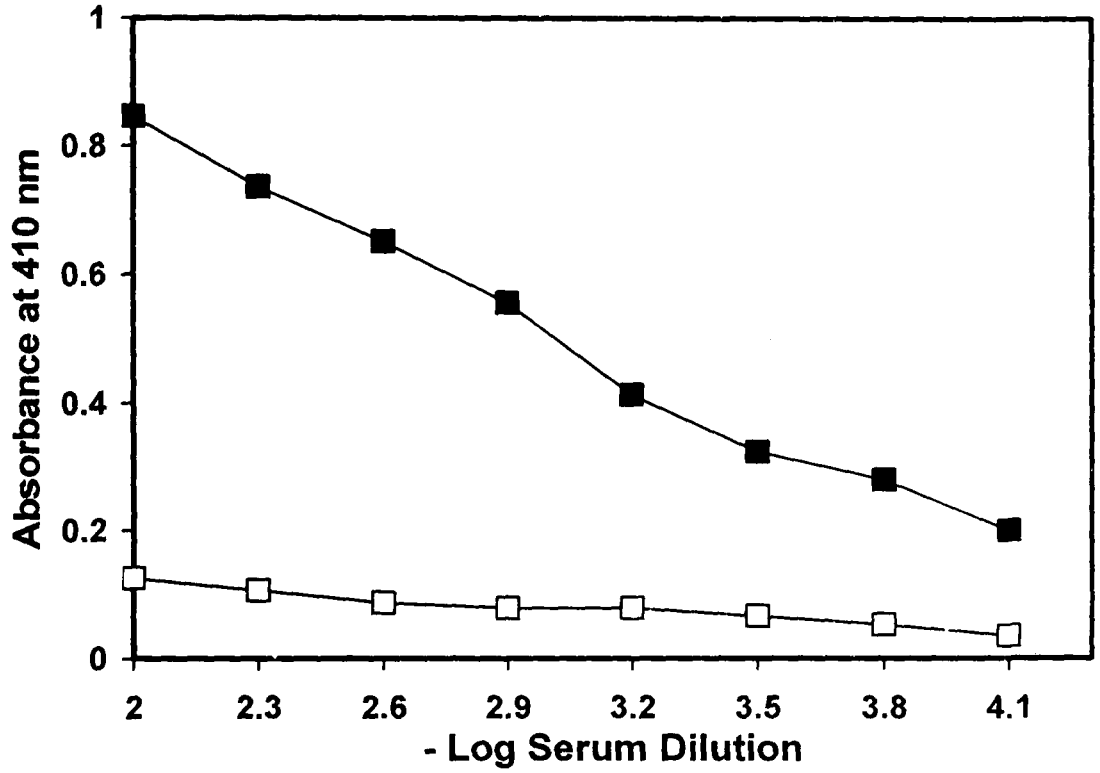


Fig. 28 Level of induced antibodies against MG-Lys-Cu^{2+} modified human DNA. Direct binding ELISA with preimmune serum (□) and immune serum (■). The microtitre wells were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).

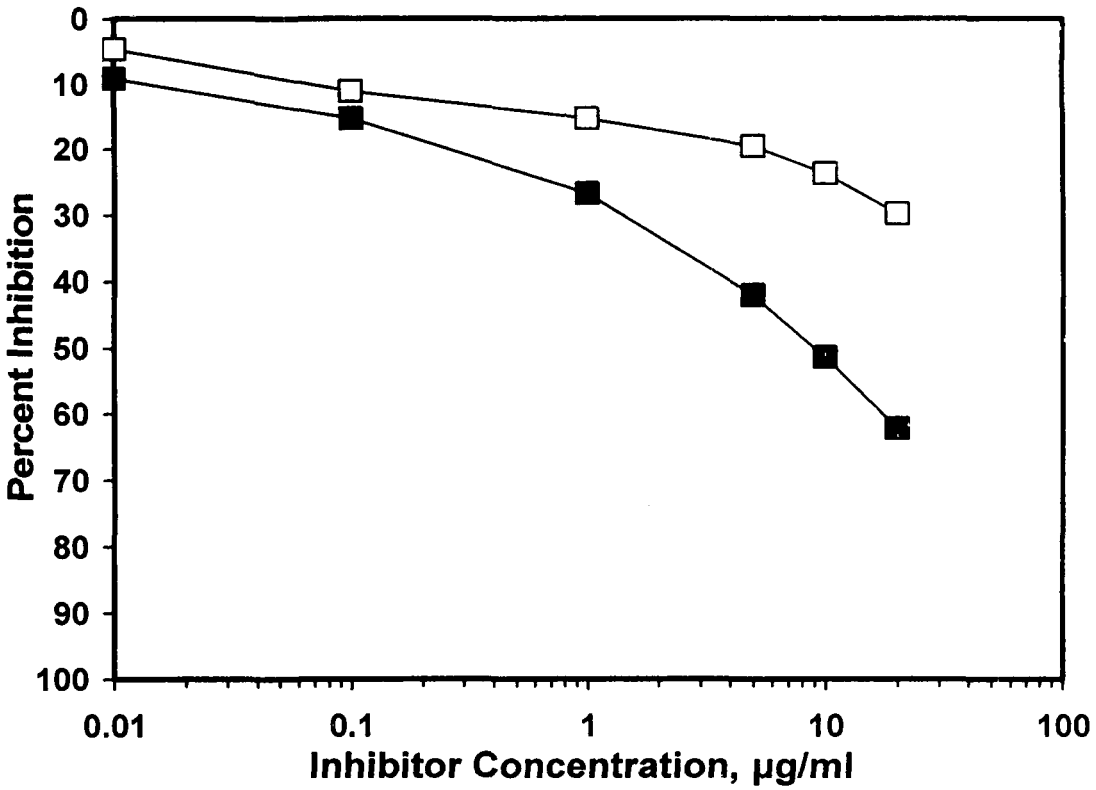


Fig. 29 Inhibition ELISA of serum antibodies against MG-Lys-Cu^{2+} modified human DNA (- ■ -) and preimmune serum (- □ -). MG-Lys-Cu^{2+} modified human DNA was used as a coating antigen (2.5 µg/ml) as well as an inhibitor.

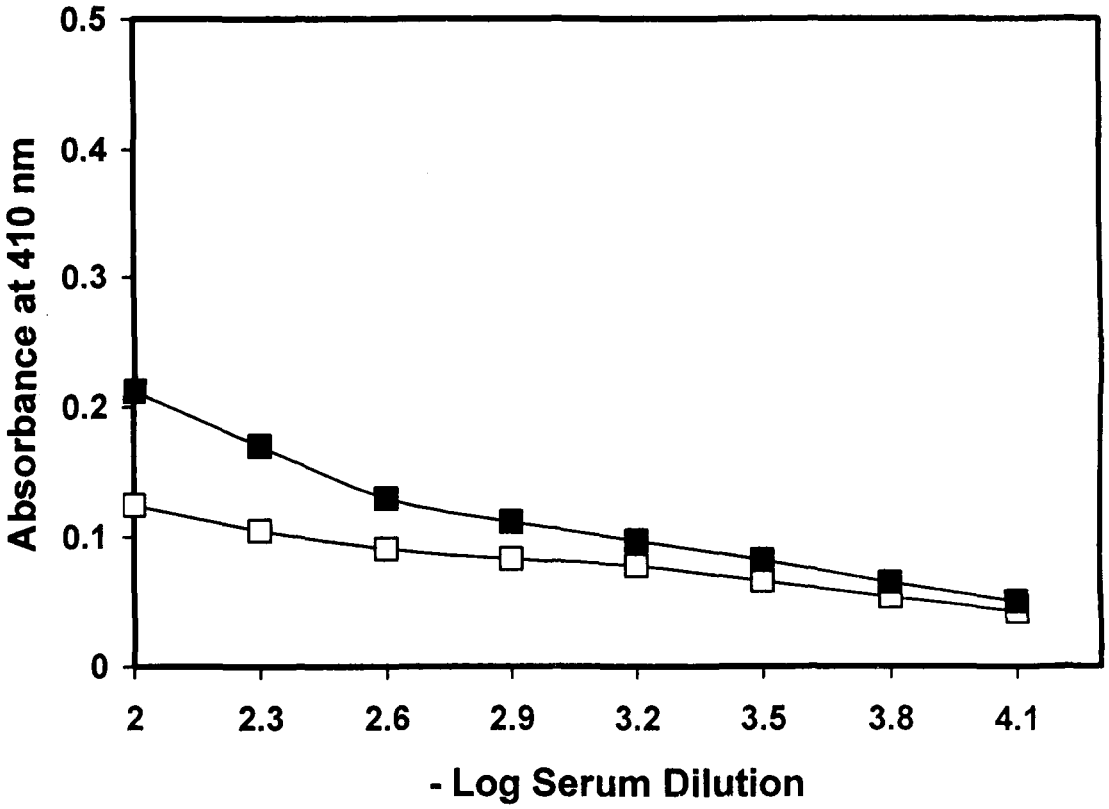


Fig. 30 Level of induced antibodies against native human DNA. Direct binding ELISA with preimmune serum (□) and immune serum (■). The microtitre wells were coated with native human DNA (2.5 μg/m

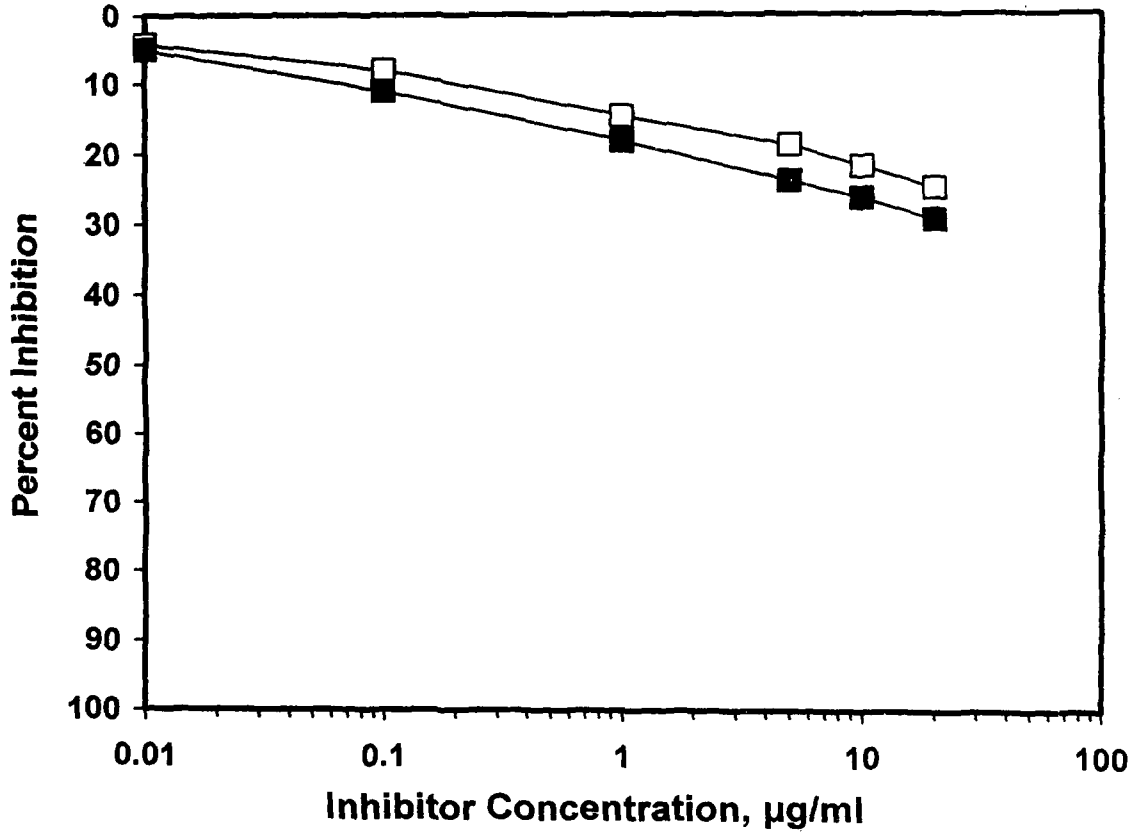


Fig. 31 Inhibition ELISA of anti-native human DNA antibodies (- ■ -) and preimmune (- □ -) sera. Native human DNA used as a coating antigen (2.5 µg/ml) as well as an inhibitor.

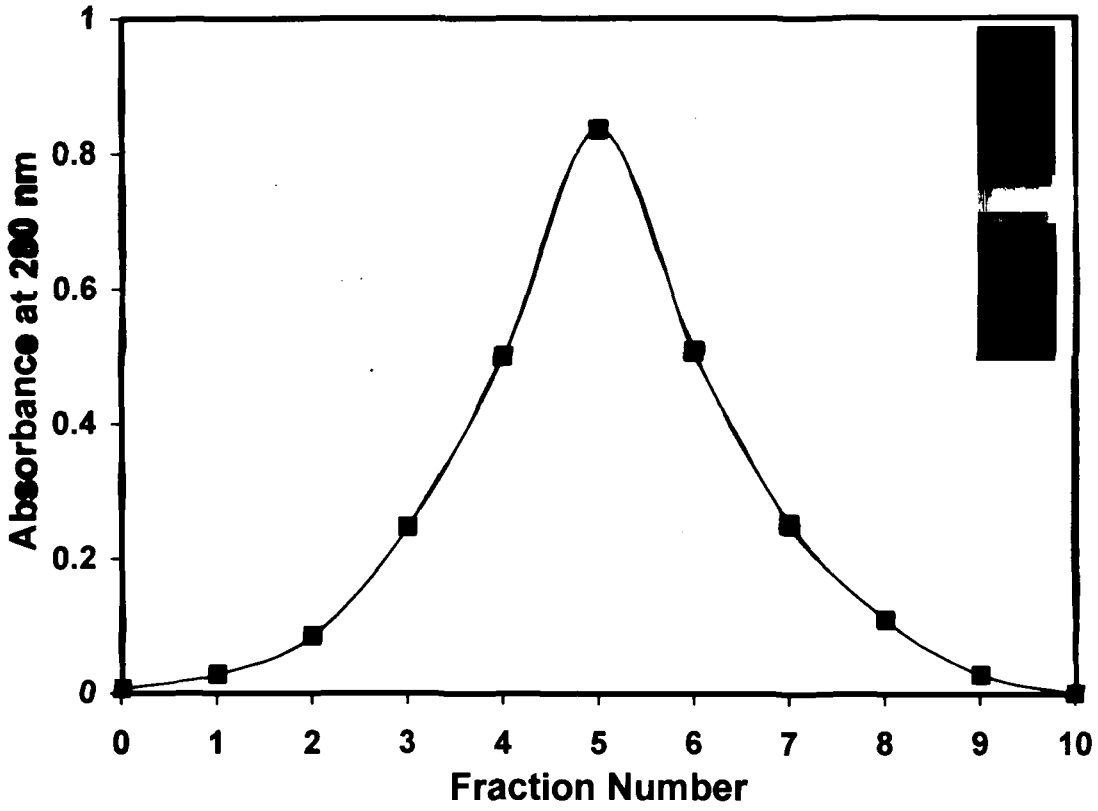


Fig. 32 Elution profile of anti-MG-Lys-Cu^{2+} modified human DNA IgG on Protein-A agarose affinity column. **Inset:** SDS-PAGE of purified IgG on 7.5% polyacrylamide ge

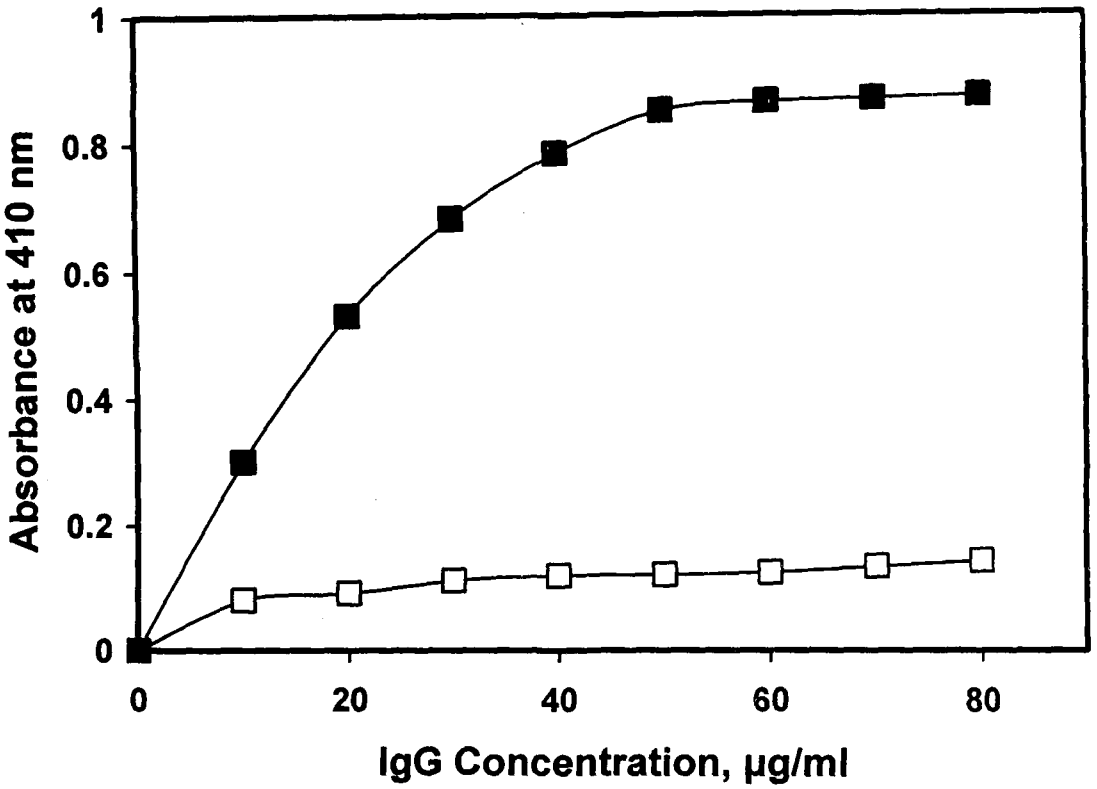


Fig. 33 Binding of affinity purified anti-MG-Lys-Cu^{2+} modified human DNA IgG (■) and preimmune IgG (□) to MG-Lys-Cu^{2+} modified human DNA. Microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

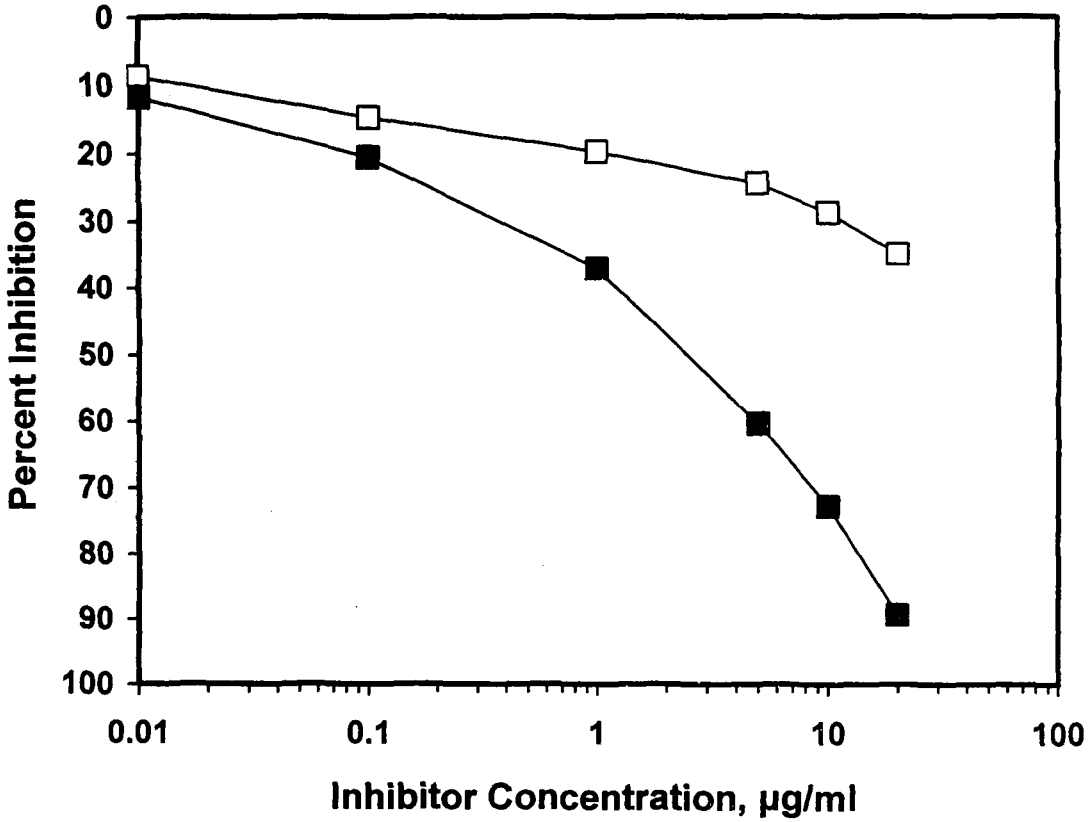


Fig. 34 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by native human DNA (- □ -) and modified human DNA (- ■ -) sera. The microtitre plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

for 2 hr at 30 mA. Figure 35 (a) shows the binding of IgG to MG-Lys-Cu^{2+} modified human DNA. With the increase in the amount of IgG, there was an increase in the formation of high molecular weight immune complexes, which resulted in retarded mobility and increased fluorescence intensity near the well. However, under similar experimental conditions the unmodified human DNA failed to show the shift in its mobility when incubated with anti-MG-Lys-Cu^{2+}-DNA IgG [Fig.35 (b)]. These results clearly indicate that epitopes on native human DNA and its modified counterpart have quite distinct recognition.

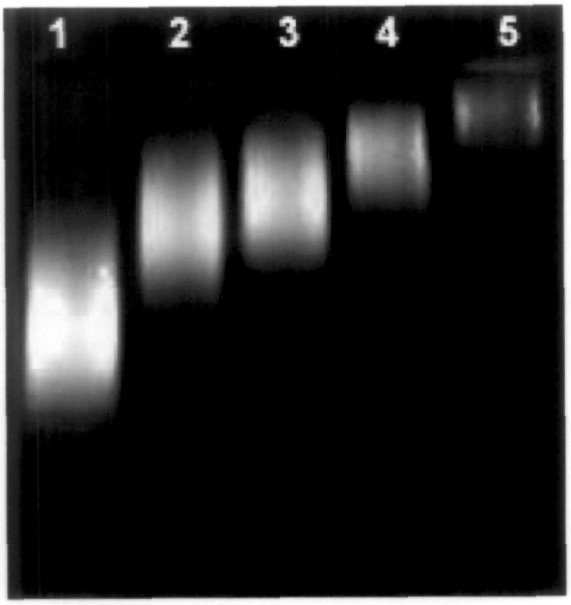
Immuno-crossreactivity of anti-MG-Lys-Cu^{2+} modified human DNA antibodies

Antigenic specificity of the induced anti-MG-Lys-Cu^{2+} modified human DNA antibodies was investigated by competitive inhibition ELISA. The induced antibodies exhibited a wide range of heterogeneity in recognizing varied inhibitors that include nucleic acid polymers and bases. A maximum of 89.5% inhibition of the anti-MG-Lys-Cu^{2+}-DNA IgG was observed with the immunogen as an inhibitor (Fig. 34). The induced antibodies were highly specific for MG-Lys-Cu^{2+} modified human DNA as only 2.8 μg/ml inhibitor was required to cause 50% inhibition. While, native human DNA caused considerably reduced inhibition (35.1 %) in antibody activity (Fig. 34).

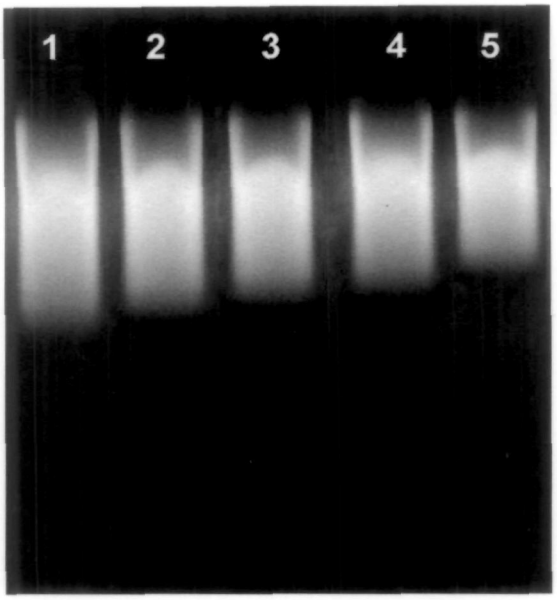
Native, ROS and MG-Lys-Cu^{2+} modified calf thymus DNA inhibited the induced antibodies by 33.3%, 48.6% and 64.2% respectively (Fig. 36). Whereas ROS modified human DNA showed maximum inhibition of 57.1% at 20 μg/ml. To achieve 50% inhibition, 13.2 μg/ml MG-Lys-Cu^{2+} modified calf thymus DNA was required. However, for ROS modified human DNA, 50% inhibition was attained at an inhibitor concentration of 15.1 μg/ml.

Plasmid DNA, its ROS and MG-Lys-Cu^{2+} modified forms caused 32.1%, 43.9% and 54.2% inhibition respectively in the binding of the immune IgG to the modified DNA (Fig. 37). The amount of MG-Lys-Cu^{2+} modified plasmid required for 50% inhibition was 19.3 μg/ml.

Lymphocyte DNA of human origin modified by MG-Lys-Cu^{2+} and ROS showed the maximum inhibition of 72.5% and 59% respectively (Fig. 38), while



(a)



(b)

Fig. 35 Band shift assay of anti-MG-Lys-Cu^{2+} modified human DNA-IgG binding to (a) MG-Lys-Cu^{2+} modified human DNA and (b) native human DNA. Electrophoresis was carried out on 0.8% agarose gel for 2 hr at 30mA.

(a) MG-Lys-Cu^{2+} modified human DNA (0.5 μg, Lane 1) was incubated with 10, 20, 40 and 60 μg of anti-MG-Lys-Cu^{2+} modified human DNA-IgG (lanes 2-5) for 2 hr at 37 ^{0}C and overnight at 4 ^{0}C.

(b) Native human DNA (0.5 μg, Lane 1) was incubated with 10, 20, 40 and 60 μg of anti-MG-Lys-Cu^{2+} modified human DNA-IgG (lanes 2-5) under identical conditions.

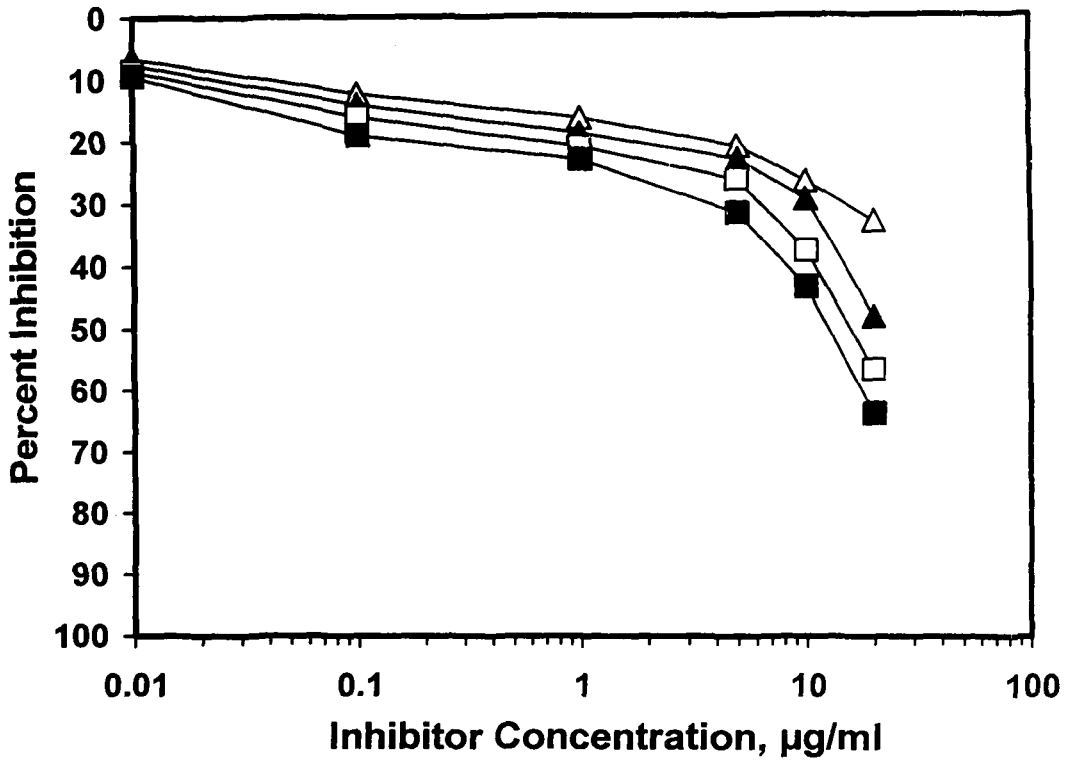


Fig. 36 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by calf thymus DNA (- Δ -), ROS modified calf thymus DNA (- ▲ -), ROS modified human DNA (- □ -) and MG-Lys-Cu^{2+} modified calf thymus DNA (- ■ -). The microtiter plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

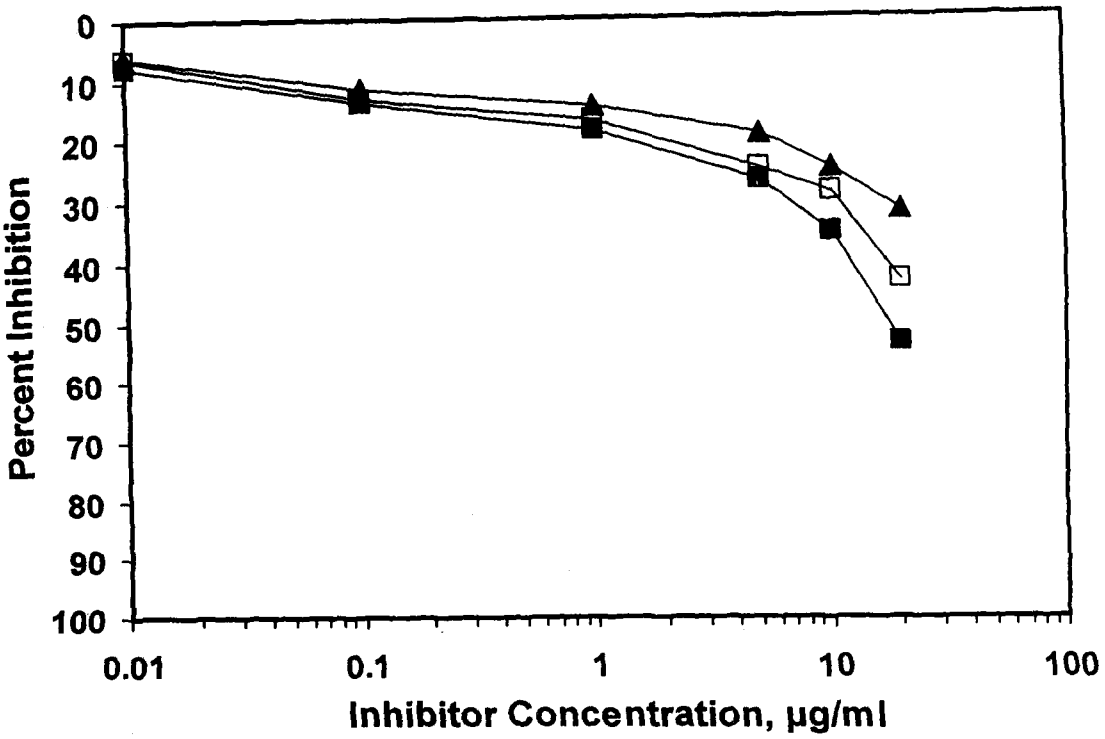


Fig. 37 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by plasmid DNA (- ▲ -), ROS modified plasmid DNA (- □ -), and MG-Lys-Cu^{2+} modified plasmid DNA (- ■ -). The microtiter plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

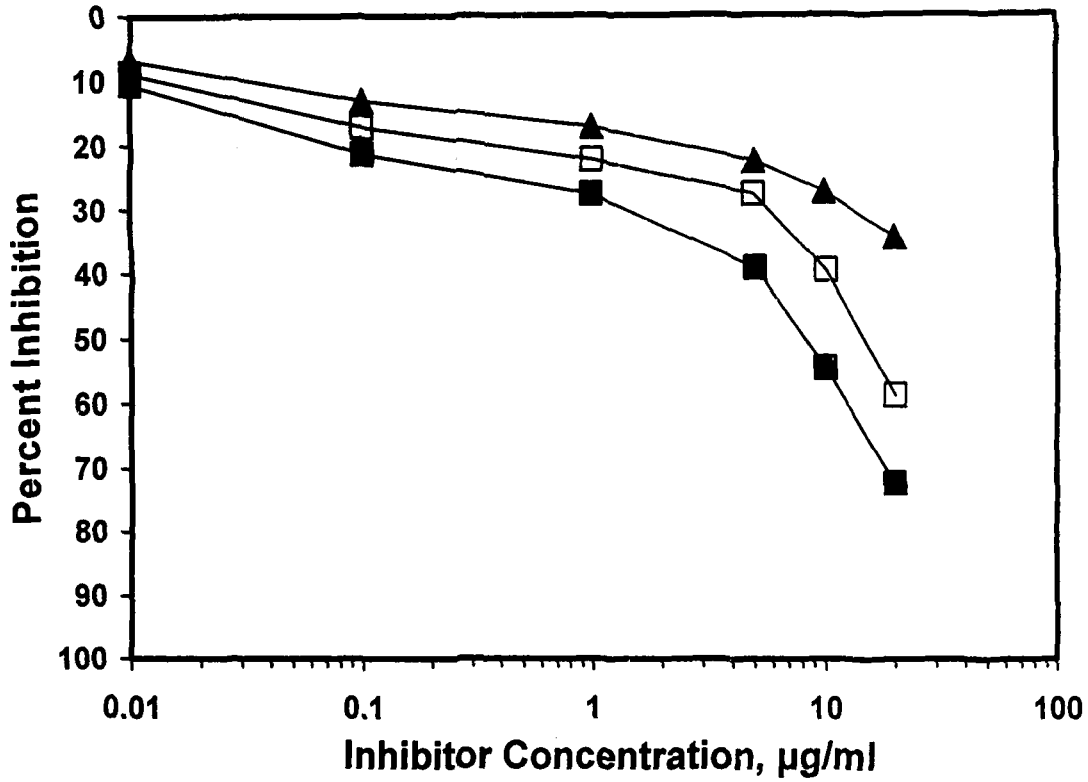


Fig. 38 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by lymphocyte DNA (- ▲ -), ROS modified lymphocyte DNA (- □ -), and MG-Lys-Cu^{2+} modified lymphocyte DNA (- ■ -). The microtiter plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

native lymphocyte DNA inhibited 34.5% binding of immune IgG to immunogen. Fifty percent inhibition of antibody activity was achieved with 8.8 μg/ml of MG-Lys-Cu^{2+} modified lymphocyte DNA and 17.8 μg/ml of ROS modified DNA.

The DNA bases guanine, cytosine, adenine, thymine and their ROS ($^{\cdot}OH$) modified and MG-Lys-Cu^{2+} modified forms were used as inhibitors of antibody binding activity. Guanine showed a moderate inhibition of 38 percent. In contrast, ROS and MG-Lys-Cu^{2+} modified guanine were potent inhibitors showing maximum inhibition of 59% and 78% respectively (Fig. 39). The amount of ROS-guanine and MG-Lys-Cu^{2+} modified guanine required for 50% inhibition in antibody activity was 16.6 μg/ml and 8.9 μg/ml respectively. Adenine caused inhibition 29% inhibition, whereas its ROS modified form was a moderate inhibitor, showing a maximum of 54% inhibition in antibody activity. In contrast, MG-Lys-Cu^{2+} modified form was found to be a potent inhibitor, showing a maximum inhibition of 65% (Fig. 40). The amount of MG-Lys-Cu^{2+} modified adenine for 50% inhibition was 14.2 μg/ml, whereas 19.4 μg/ml was required in the case of ROS-modified adenine to get 50 % inhibition in the antibody activity. Thymine and its ROS-modified form produced 26% and 32% inhibition respectively (Fig. 41), whereas MG-Lys-Cu^{2+}-thymine showed a moderate inhibition of 43%. In contrast, cytosine and its ROS and MG-Lys-Cu^{2+} modified forms showed a maximum of 27%, 43% and 47% inhibition respectively (Fig. 42). Binding characteristics of the induced antibodies against MG-Lys-Cu^{2+}- DNA have been summarized in Table 6.

Binding of autoantibodies against native and MG-Lys-Cu^{2+} modified human DNA in diabetes patients

In this clinical study, we have randomly selected total 85 cases of both diabetes type I (40 samples) and type II (45 samples). The sera were obtained from patients attending J.N. Medical College and hospital, A.M.U., Aligarh after the informed consent. Sera from age and sex-matched normal healthy individuals served as control. All sera were diluted to 1:100 in TBS-T and subjected to direct binding ELISA on solid phase separately coated with equal amounts of human DNA and MG-Lys-Cu^{2+} modified human DNA. Out of total 40 type I diabetes sera, 27 samples

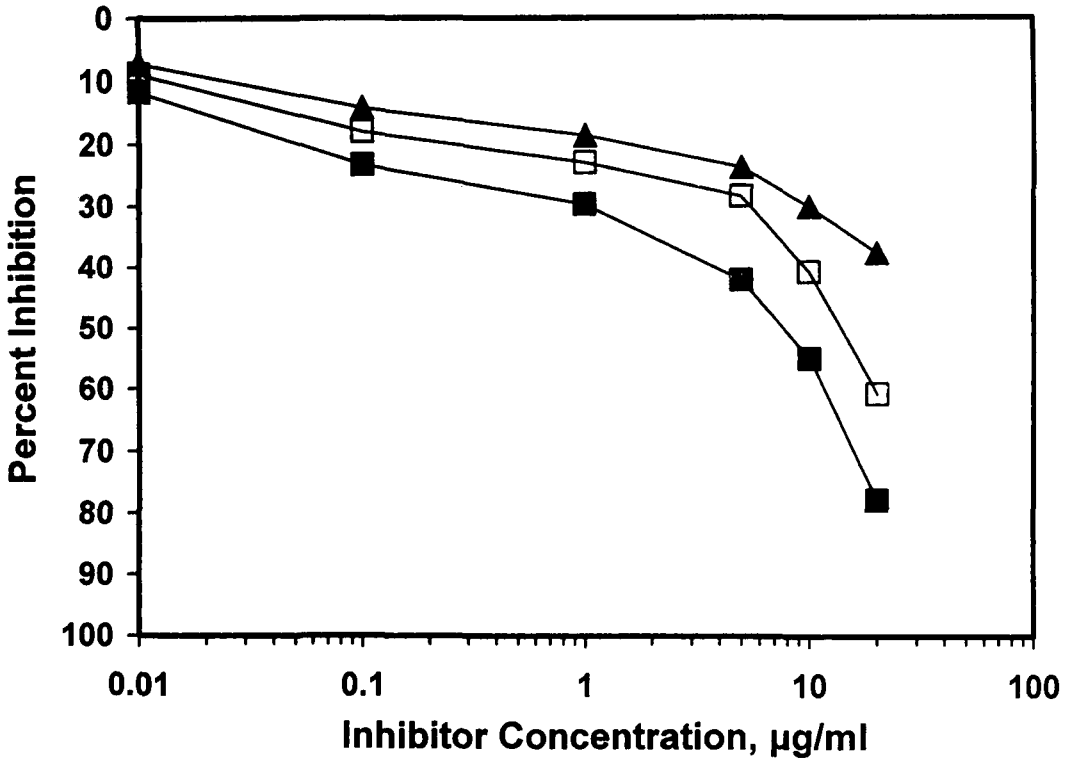


Fig. 39 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by guanine (- ▲ -), ROS modified guanine (- □ -), and MG-Lys-Cu^{2+} modified guanine (- ■ -). The microtiter plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

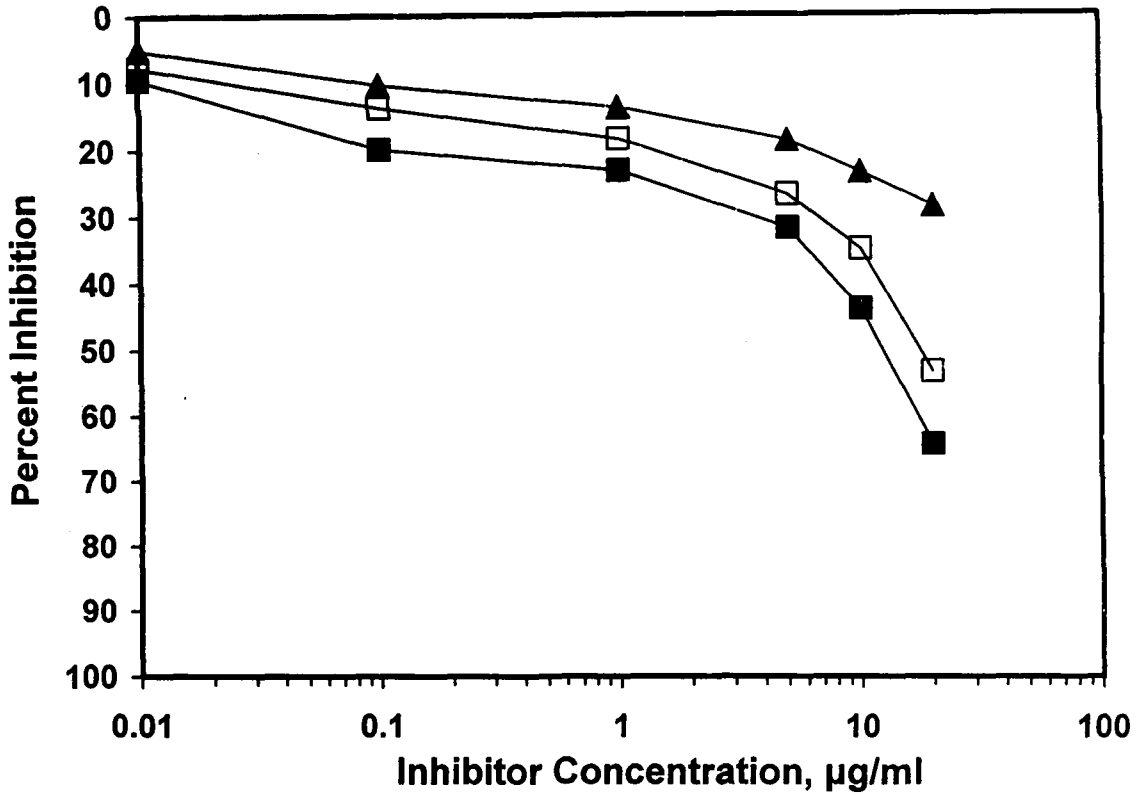


Fig. 40 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by adenine (- ▲ -), ROS modified adenine (- □ -), and MG-Lys-Cu^{2+} modified adenine (- ■ -). The microtiter plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

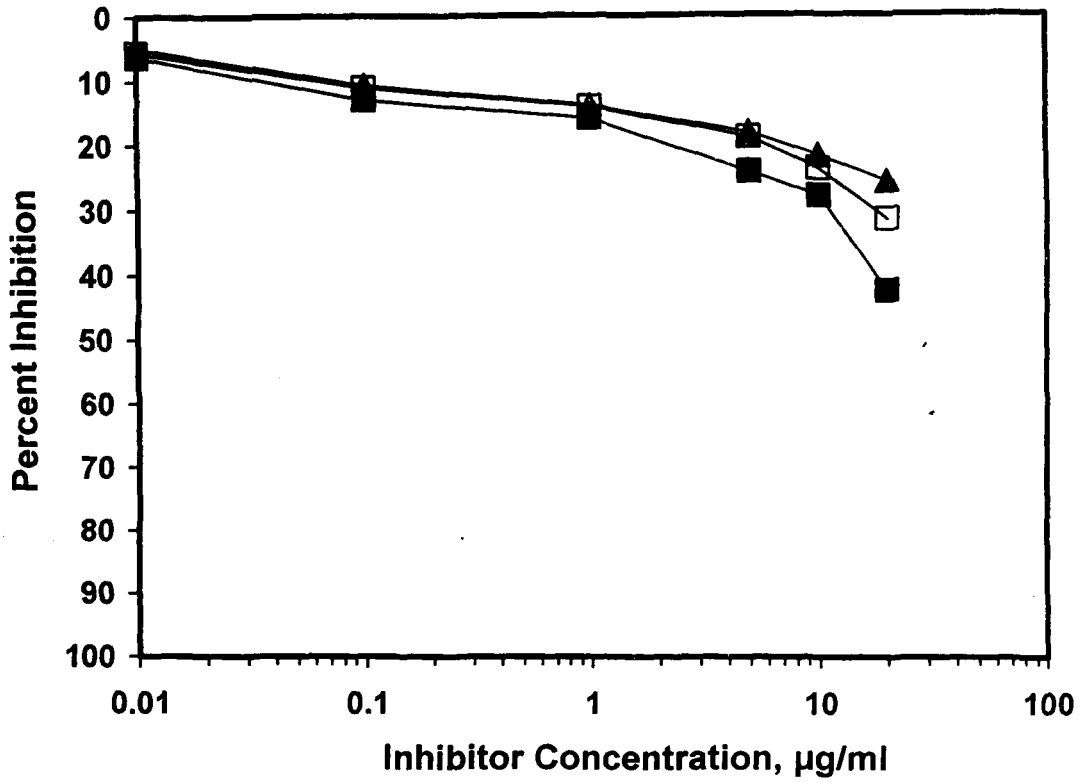


Fig. 41 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by thymine (- ▲ -), ROS modified thymine (- □ -), and MG-Lys-Cu^{2+} modified thymine (- ■ -). The microtiter plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml)

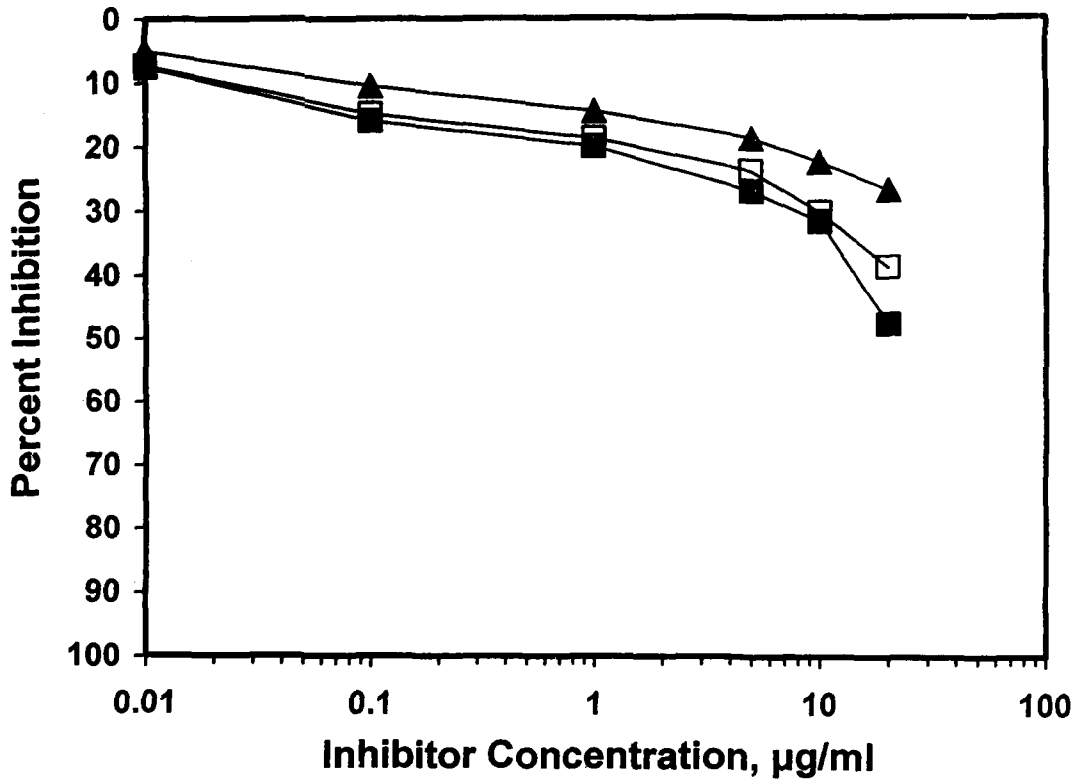


Fig. 42 Inhibition of anti-MG-Lys-Cu^{2+} modified human DNA IgG binding by cytosine (- ▲ -), ROS modified cytosine (- □ -), and MG-Lys-Cu^{2+} modified cytosine (- ■ -). The microtiter plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

TABLE 6

Cross-reaction of anti-MG-Lys-Cu^{2+} modified human DNA IgG

Inhibitors	Maximum% inhibition at 20 µg/ml	Concentration for 50%inhibition (µg/ml)	Percent relative affinity
MG-Lys-Cu^{2+}- human DNA	89.5	2.8	100
Native human DNA	35.1	-	-
Native calf thymus DNA	33.3	-	-
ROS calf thymus DNA	48.6	-	-
ROS human DNA	57.1	15.1	18.5
MG-Lys-Cu^{2+}- calf thymus DNA	64.2	13.2	21.2
Native plasmid	32.1	-	-
ROS plasmid	43.9	-	-
MG-Lys-Cu^{2+}-plasmid	54.2	19.3	14.5
Native human lymphocyte DNA	35	-	-
ROS human lymphocyte DNA	59	17.8	15.7
MG-Lys-Cu^{2+}- human lymphocyte DNA	72.5	8.8	31.8
Native guanine	38	-	-
ROS guanine	60	16.6	16.8
MG-Lys-Cu^{2+}-guanine	78	8.9	31.4
Native adenine	29	-	-
ROS adenine	54	19.4	14.4
MG-Lys-Cu^{2+}-adenine	65	14.2	19.7
Native thymine	26	-	-
ROS thymine	32	-	-
MG-Lys-Cu^{2+}-thymine	43	-	-
Native cytosine	27	-	-
ROS cytosine	39	-	-
MG-Lys-Cu^{2+}-cytosine	48	-	-

Microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5µg/ml).

67.5%), (Figs. 43-46) showed higher binding with MG-Lys-Cu^{2+} modified human DNA, while in type II diabetes only 16 sera (35.5%) out of 45 samples tested, showed enhanced binding with modified human DNA as compared to the native form.

The serum samples which showed enhanced binding were considered for further studies, while samples whose absorbance was less than or equal to control were not included.

Immuno-crossreactivity of autoantibodies from diabetes type I patients

Competition ELISA was carried out to analyze the specific binding of circulating autoantibodies in type I diabetic patients sera for native and MG-Lys-Cu^{2+} modified human DNA.

In the 27 sera chosen from type I diabetes patients, which showed enhanced binding, the observed maximum inhibition with MG-Lys-Cu^{2+} modified human DNA was in the range of 46.9 to 63.1% while with native human DNA it ranged from 22 to 33% (Figs. 47-55). Mean inhibition for the entire sample tested with native human DNA was 27±3.8%, while for MG-Lys-Cu^{2+} modified human DNA, it was 54.95±5.4%. The above results have been summarized in Table 7.

Purification of IgG from the sera of type I diabetes patient

IgG was isolated on a protein A-agarose column from selected high binding sera of type I diabetes patients. The purified IgG eluted as a symmetrical single peak on the affinity column (Fig. 56). IgG purity was confirmed by a single homogeneous band on SDS-PAGE under non-reducing conditions (Fig. 56 inset).

Binding of IgG from different diabetes type I patients to native and MG-Lys-Cu^{2+} modified human DNA

Purified IgG from type I diabetes patients, were subjected to direct binding ELISA, on a microtitre plate coated with native human DNA and MG-Lys-Cu^{2+} modified human DNA to evaluate the amount required for antigen saturation. The saturation for modified human DNA was obtained at 50 μg/ml of IgG, while for native human antigenic saturation could not be ascertained because of its negligible

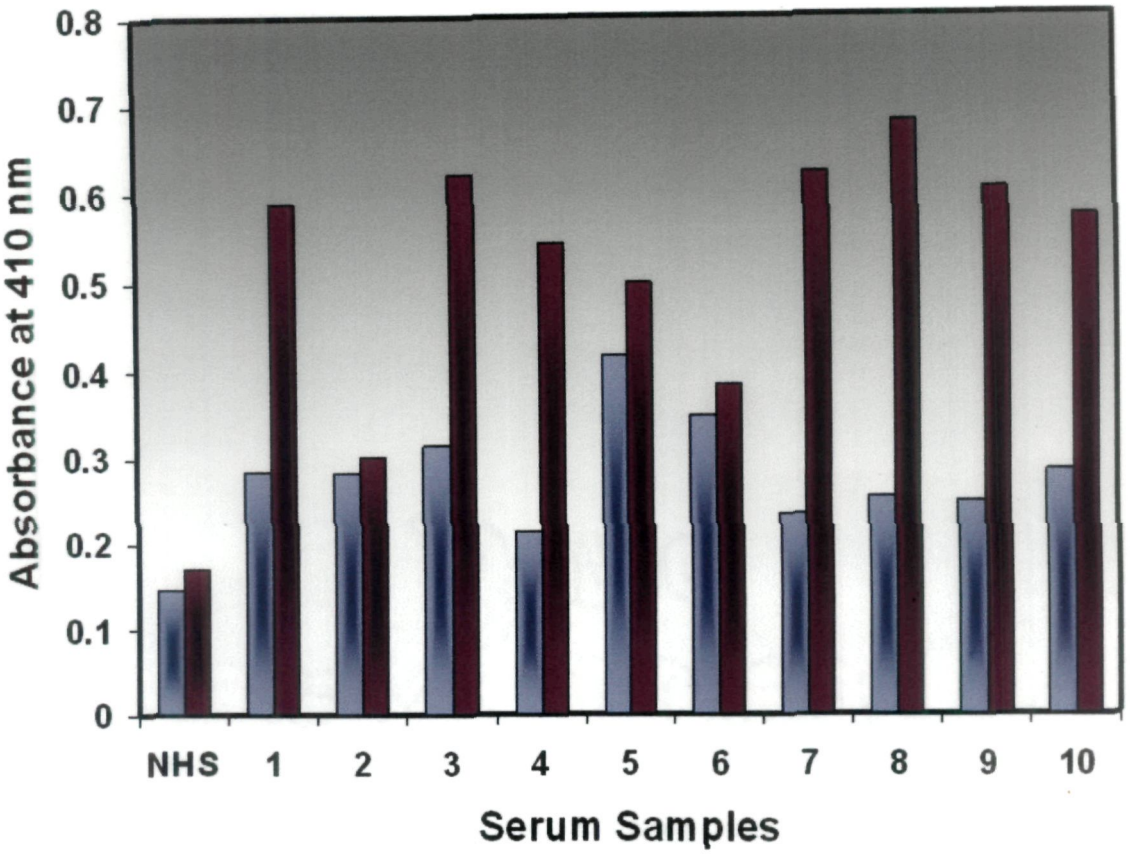


Fig. 43 Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 1-10) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml)

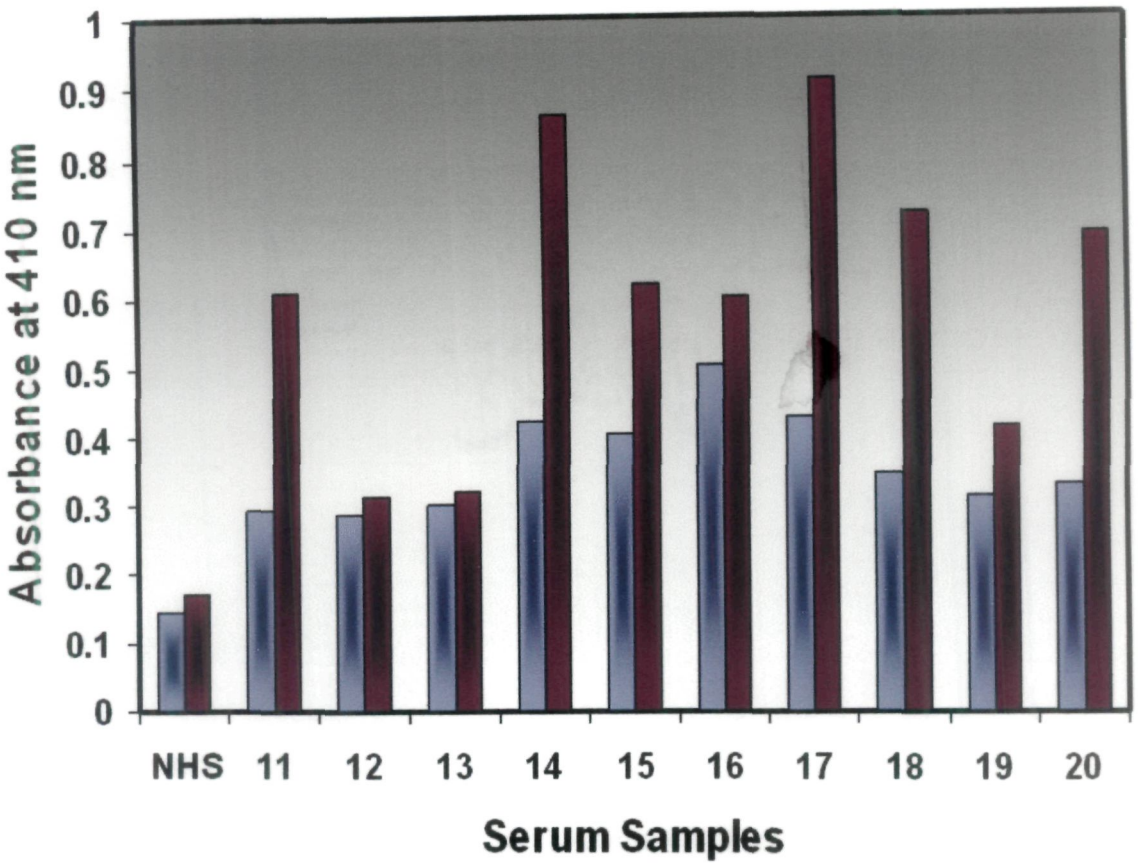


Fig. 44 Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 11-20) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).

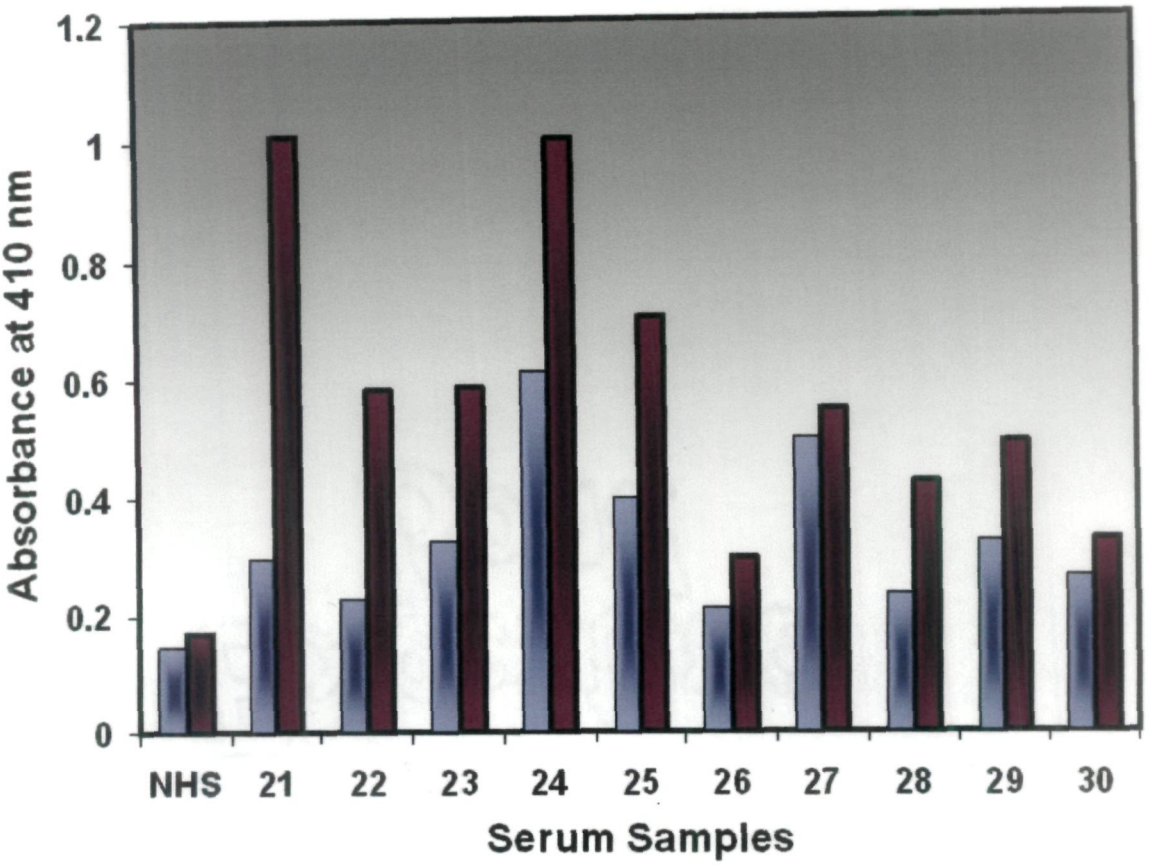


Fig. 45 Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 21-30) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

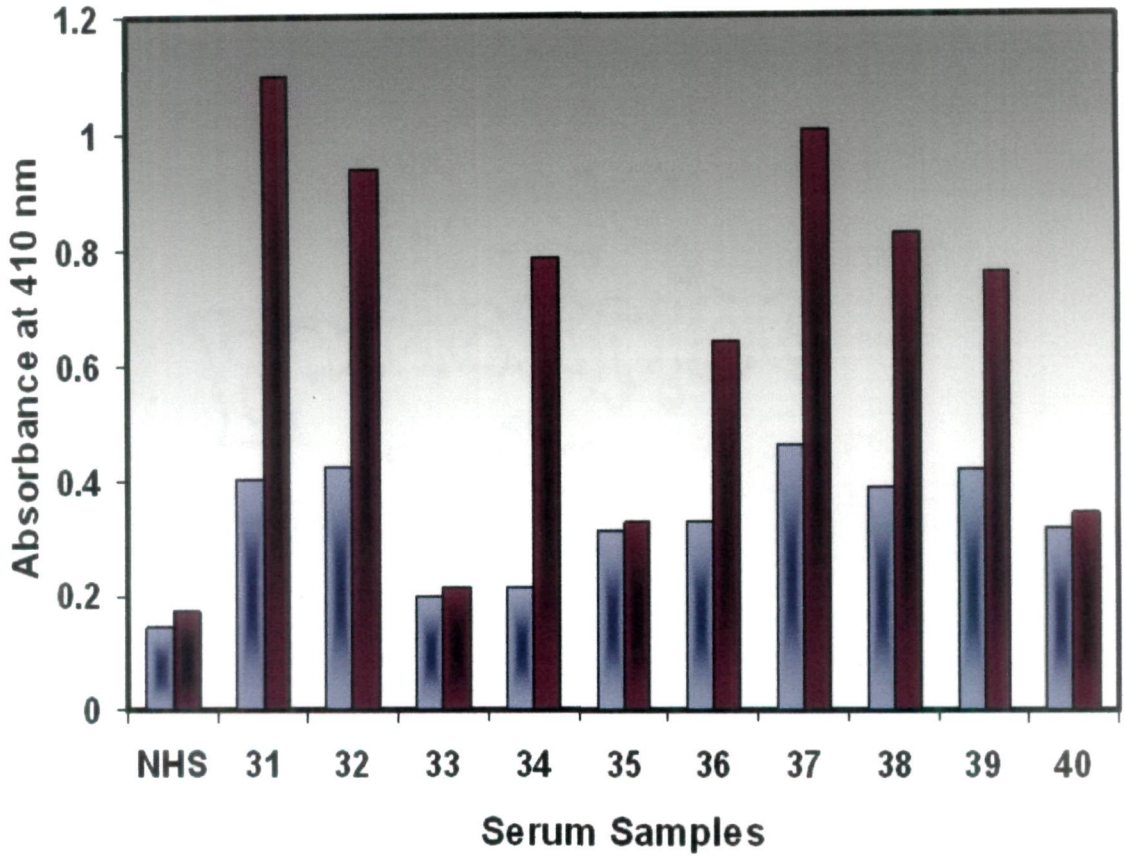


Fig. 46 Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 31-40) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).

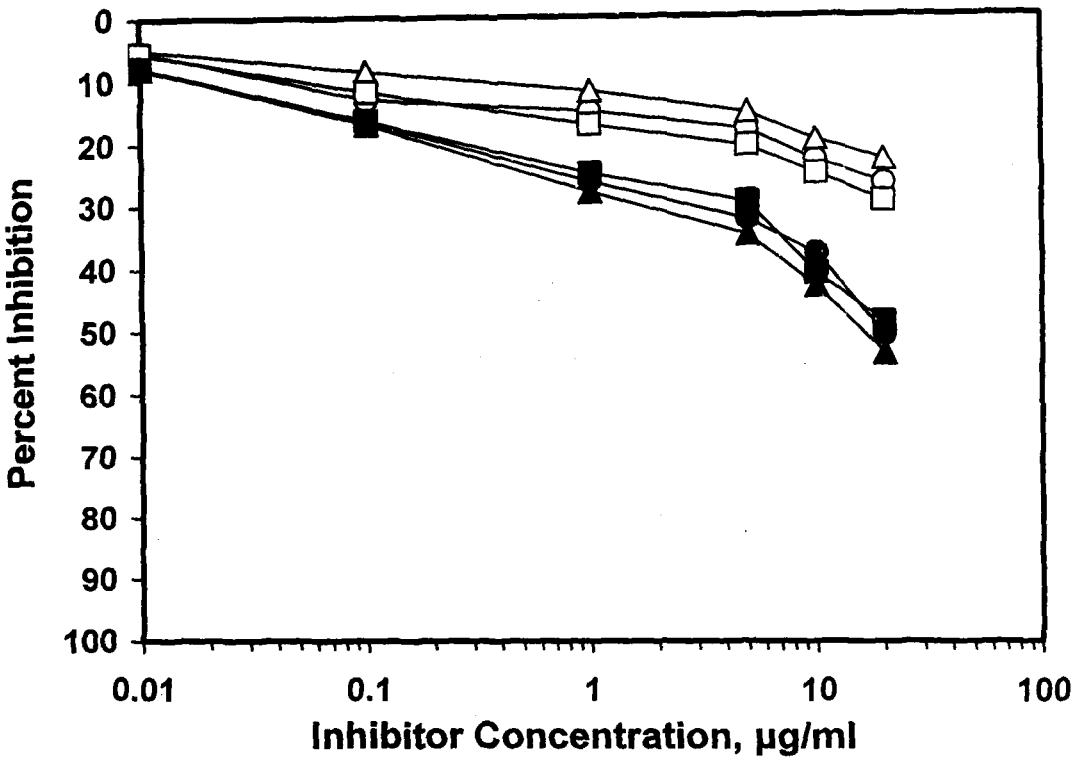


Fig. 47 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (●, ▲, ■). Sera 1, 3 and 4 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

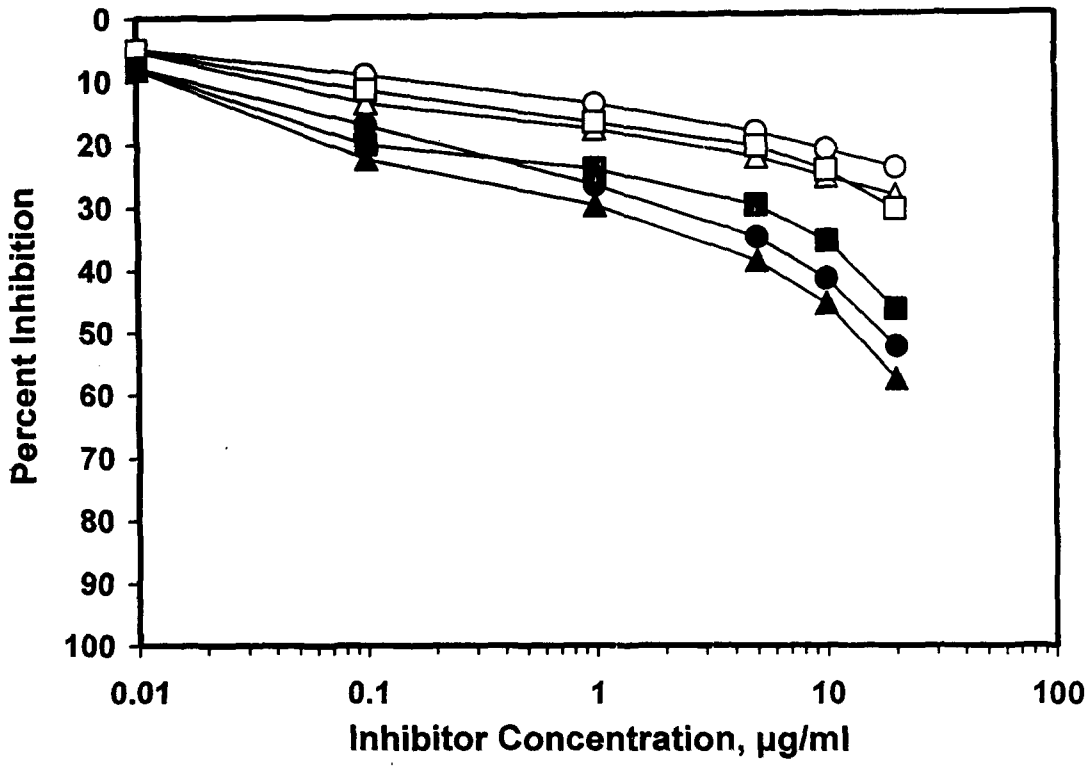


Fig. 48 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (●, ▲, ■). Sera 7, 8 and 9 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

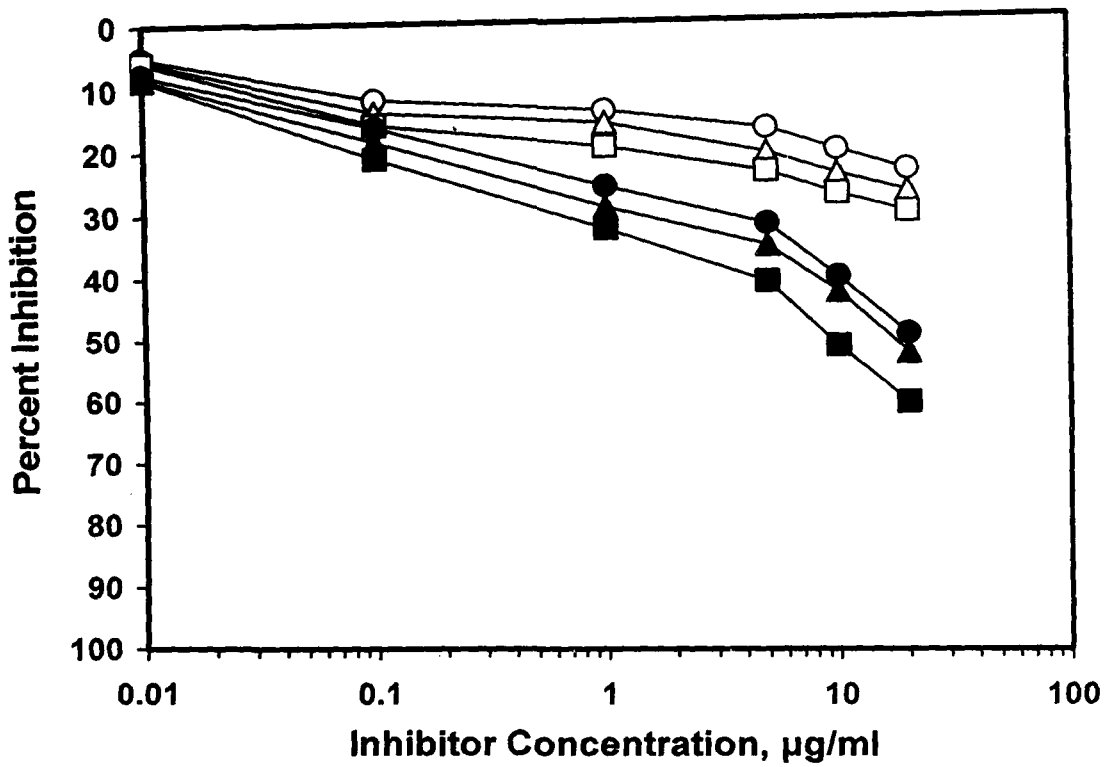


Fig. 49 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 10, 11 and 14 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

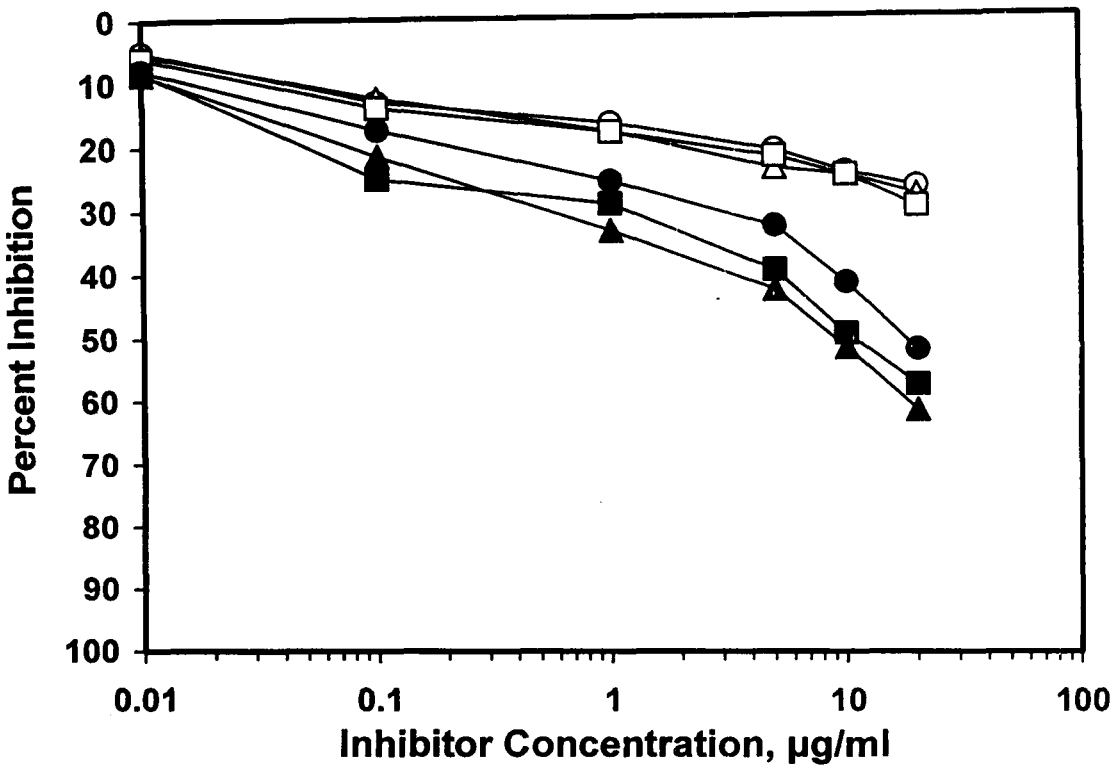


Fig. 50 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 15, 17 and 18 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

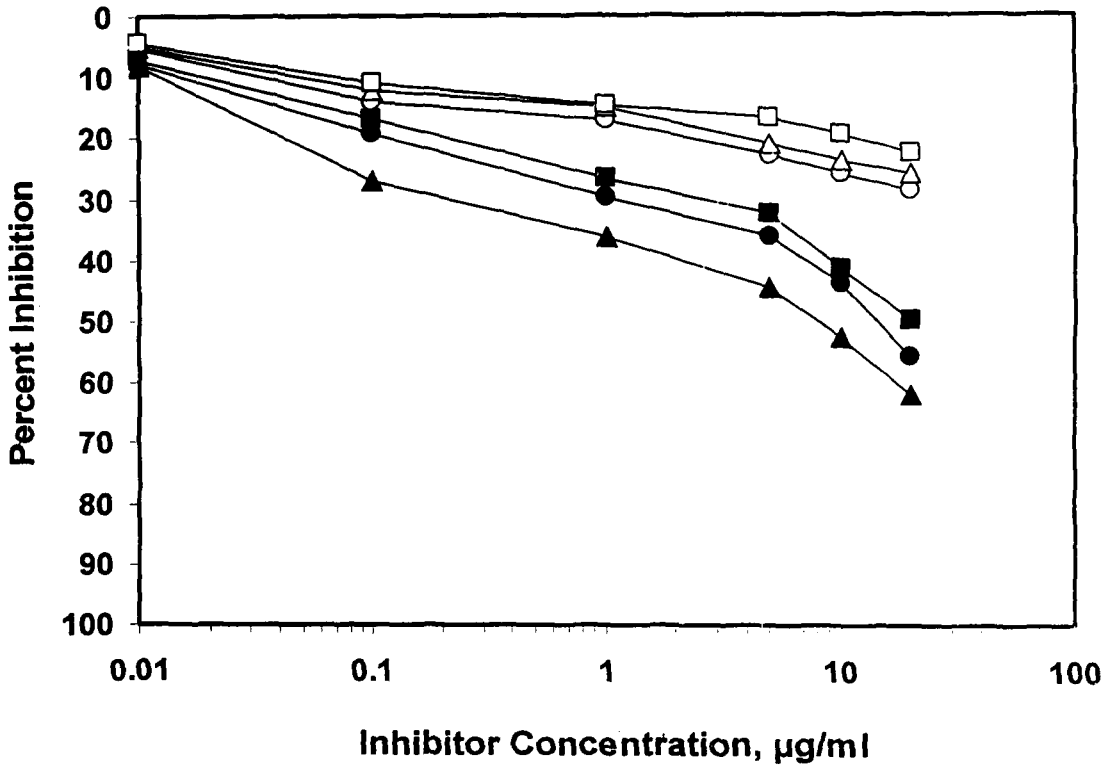


Fig. 51 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 20, 21 and 22 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

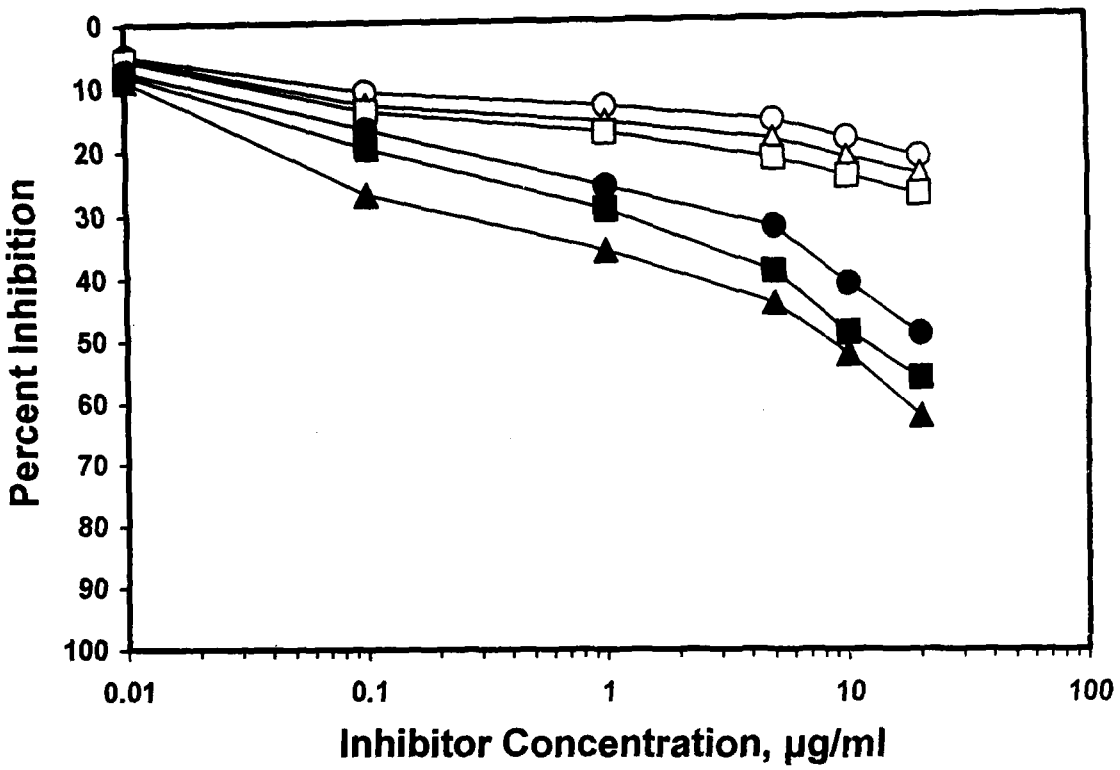


Fig. 52 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 23, 24 and 25 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

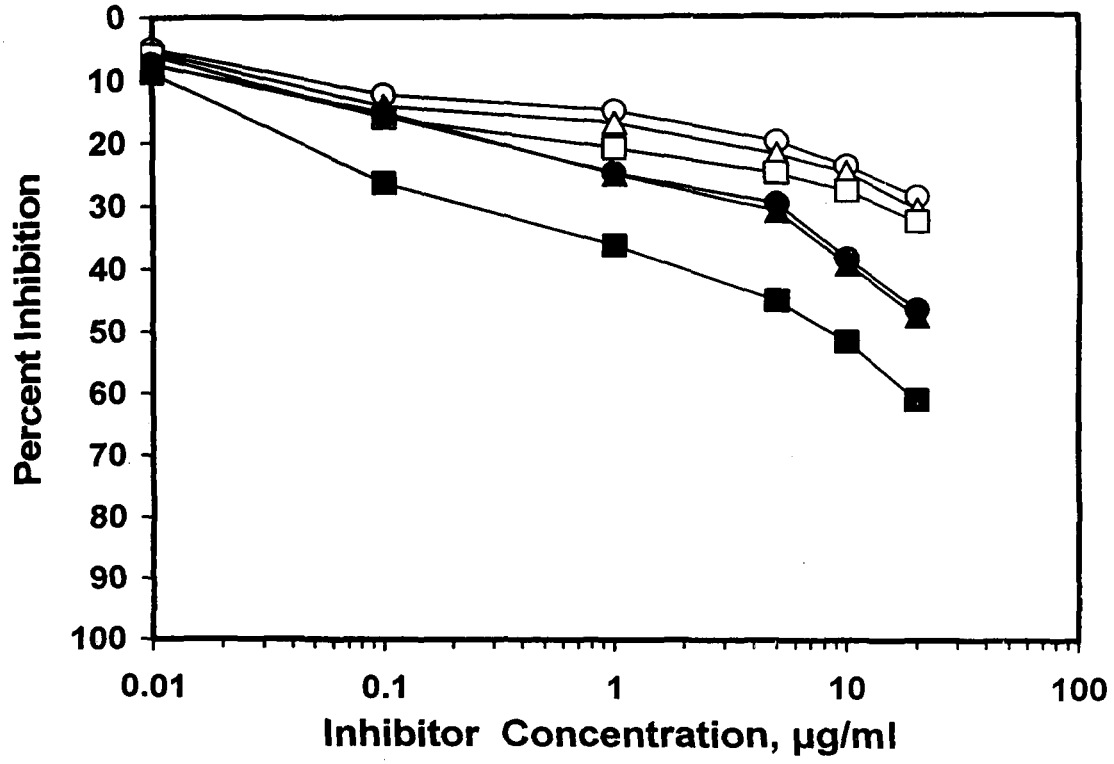


Fig. 53 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 28, 29 and 31 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml)

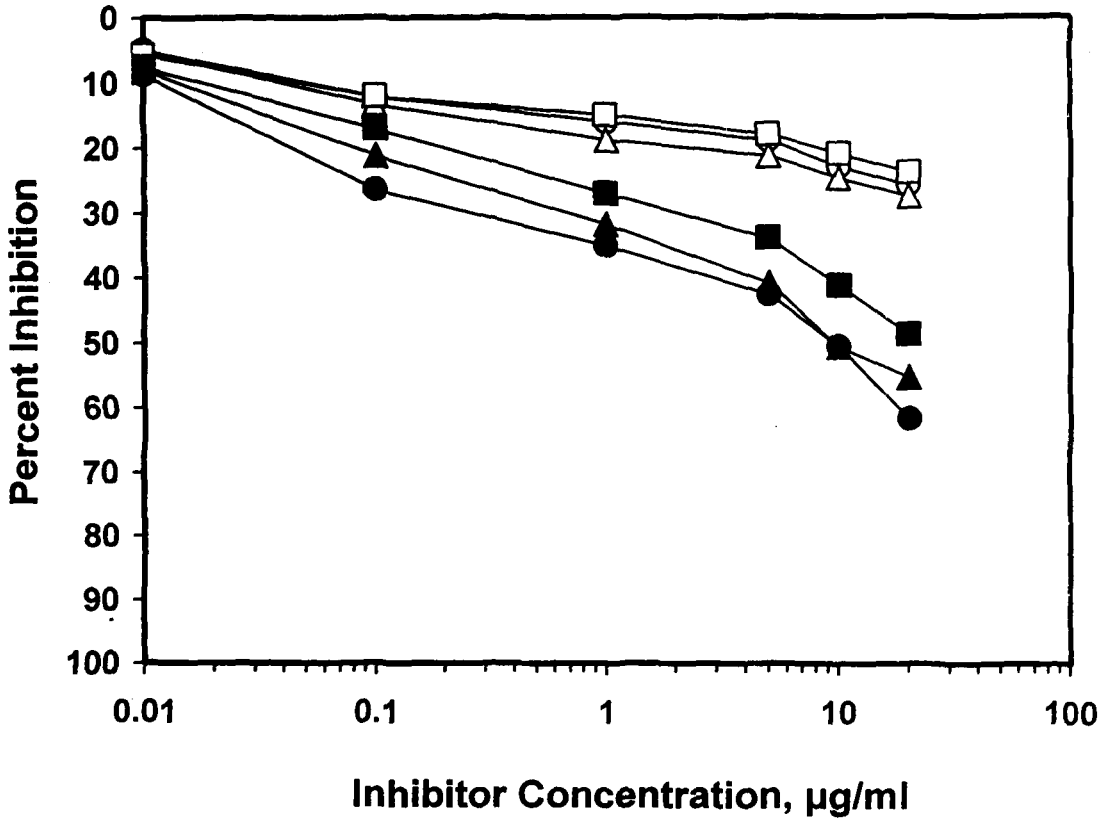


Fig. 54 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 32, 34 and 36 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

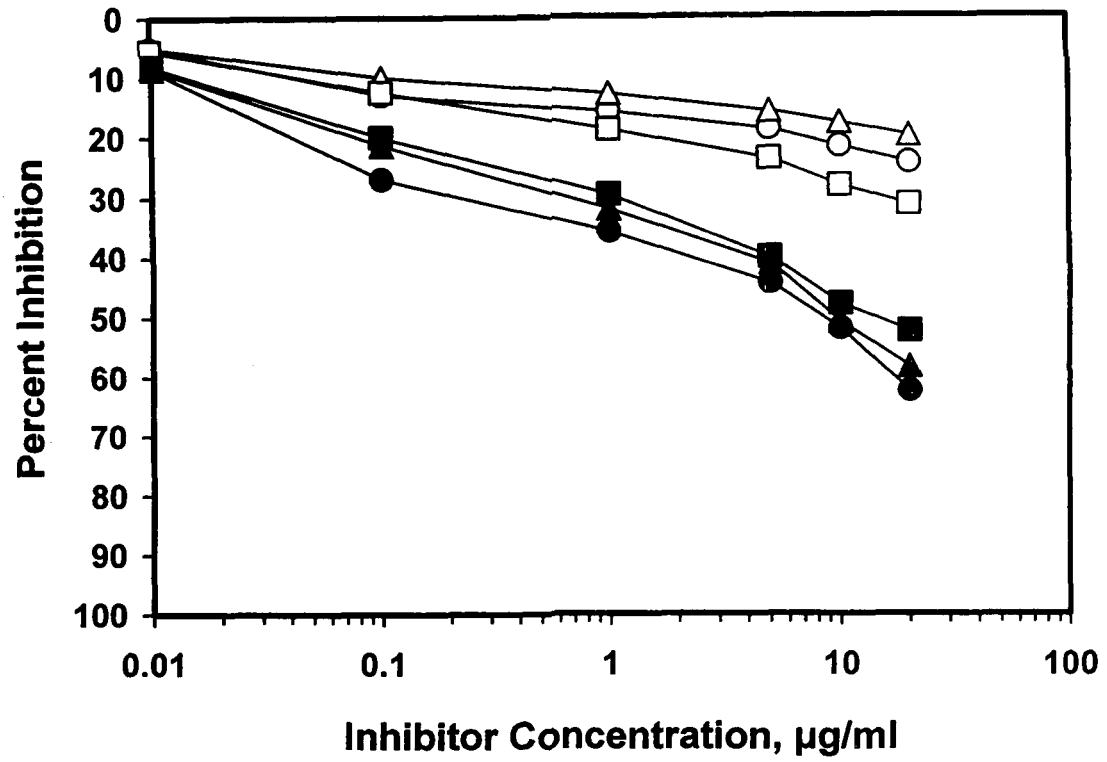


Fig. 55 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 37, 38 and 39 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

TABLE 7

Competitive inhibition data of serum autoantibodies in type I diabetes patients

Sera no	Maximum percent inhibition at 20 μg/ml	
	Native human DNA	MG-Lys-Cu^{2+} modified human DNA
01	26.8	51.0
03	22.9	54.0
04	29.6	49.0
07	24.5	52.8
08	28.8	58.0
09	31.0	46.9
10	24.0	50.0
11	27.4	53.0
14	30.7	61.0
15	26.8	52.5
17	28.0	62.0
18	30.0	58.0
20	29.0	54.0
21	26.0	62.0
22	20.8	48.0
23	22.0	50.0
24	24.8	63.1
25	28.0	57.0
28	29.0	47.0
29	31.0	48.0
31	33.0	61.3
32	26.0	62.0
34	27.8	55.7
36	24.0	49.1
37	24.7	62.8
38	20.2	58.6
39	31.7	52.8
Mean ± SD	26.98±3.8%	54.8±5.4%

The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5μg/ml).

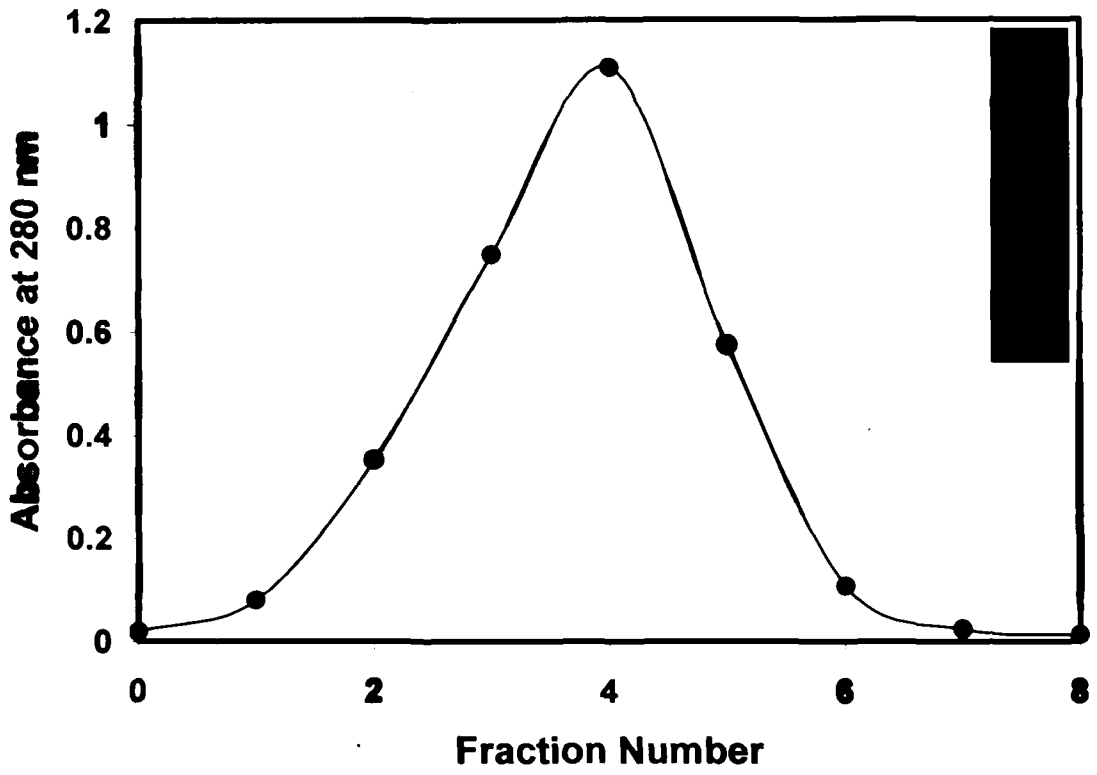


Fig. 56 Elution profile of IgG from the serum sample of diabetes type I patients on Protein A-agarose affinity column. Inset: SDS- PAGE of purified IgG on 7.5% polyacrylamide gel. Similar pattern was obtained in all sera.

binding. Therefore, for type I diabetes, IgG concentration was kept constant (50 μg/ml) in all further experiments unless indicated.

The binding specificity of the isolated IgG, towards native and MG-Lys-Cu^{2+} modified human DNA, was evaluated by inhibition ELISA. The IgG was mixed with varying amounts of native or MG-Lys-Cu^{2+} modified human DNA (0-20 μg/ml) and incubated for 2 hr at 37 °C and overnight at 4 °C. The inhibition results of type I diabetic sera IgG have been presented in Figs. 57-61. The observed antibody (IgG) inhibition ranged from 59-69% when modified human DNA was employed as inhibitor, while with the native human DNA it varied from 27-36%; maximum inhibitor concentration being 20 μg/ml in both the cases. The mean of inhibitions for various sample tested with the MG-Lys-Cu^{2+} modified human DNA was 66.2±7.1%, while with native human DNA, it was 31.3±3.2%. Table 8 summarizes the inhibition data of isolated IgG of diabetes type I group.

Band shift assay

Band shift assay was performed for the visual detection of interaction of native and MG-Lys-Cu^{2+} modified human DNA with purified IgG from type I diabetes patients IgG. Constant amount of antigens were incubated with increasing concentration of IgG for 2 hr at 37 °C and overnight at 4 °C, resulted in a proportional increase in the formation of high molecular weight immune complexes as visualized by retarded mobility and gradually increased band intensity near the wells in agarose gel electrophoresis. Normal human IgG incubated under identical conditions did not show immune complex formation (Fig. 62 a & b).

Detection of antibodies against native and MG-Lys-Cu^{2+} modified human DNA in diabetes type II patients

Forty five samples from diabetes type II patients were analysed for native and MG-Lys-Cu^{2+} modified human DNA by direct binding ELISA. Sera from normal healthy individuals served as controls. Out of 45 serum sample only 16 sera (35.5%), exhibited enhanced binding with MG-Lys-Cu^{2+} modified human DNA as compared to the native DNA (Figs. 63-66). However, the binding in diabetes type II patients was found appreciably lower than in type I patients. The specific binding of serum

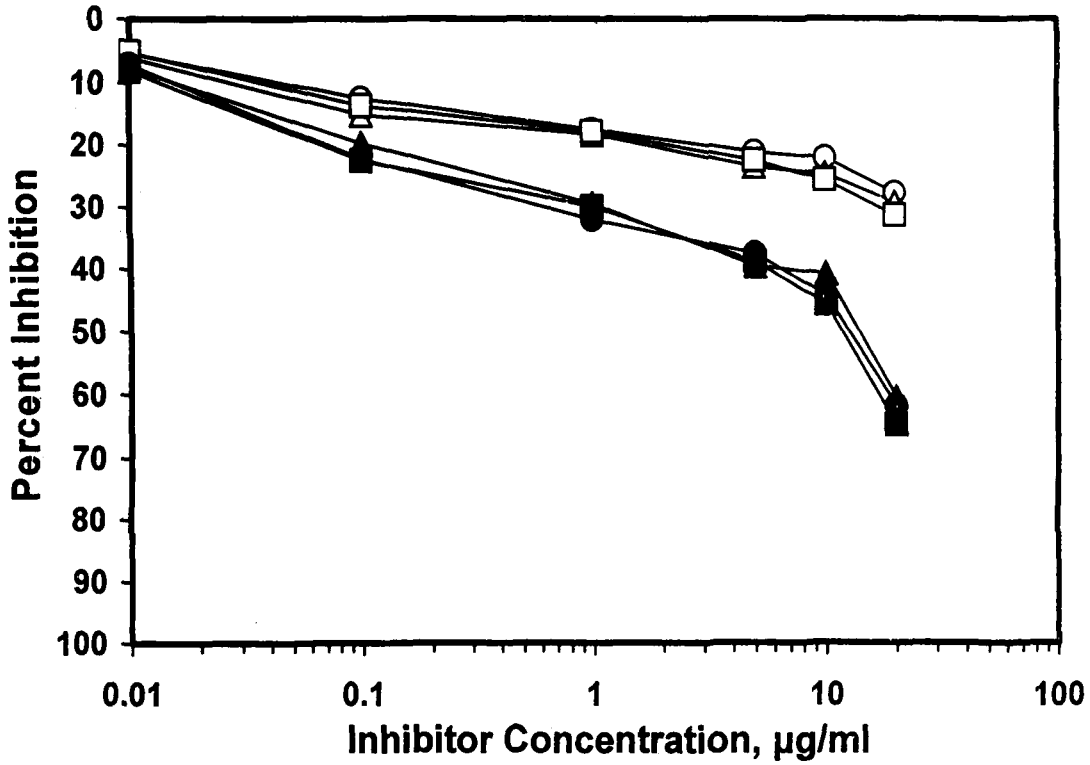


Fig. 57 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml). The above plot shows the inhibition in IgG isolated from serum samples 3, 7 and 8.

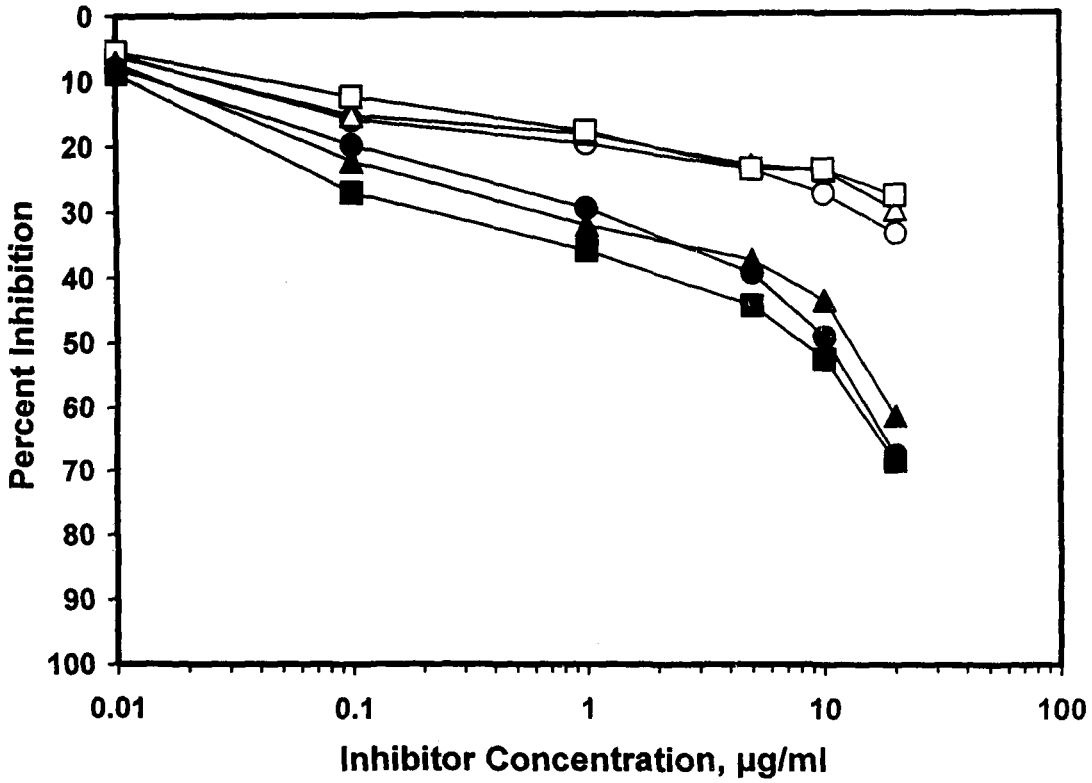


Fig. 58 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml). The above plot shows the inhibition in IgG isolated from serum samples 14, 15 and 17.

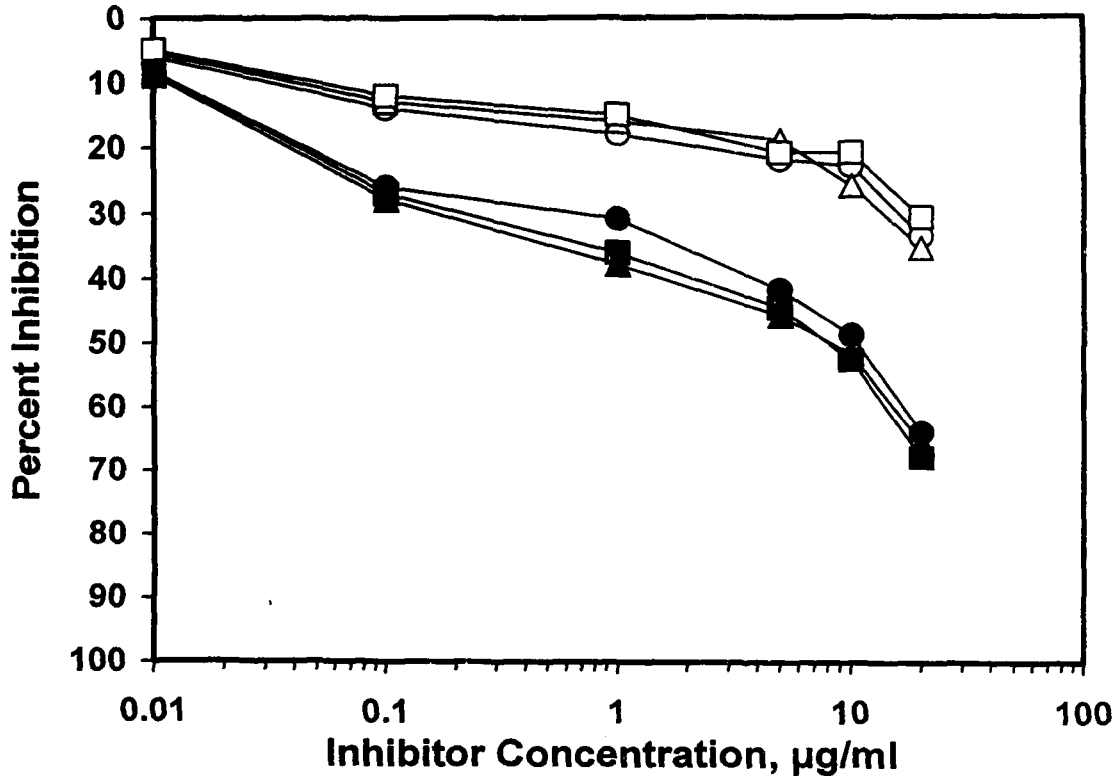


Fig. 59 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (●, ▲, ■). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml). The above plot shows the inhibition in IgG isolated from serum samples 18, 21 and 24.

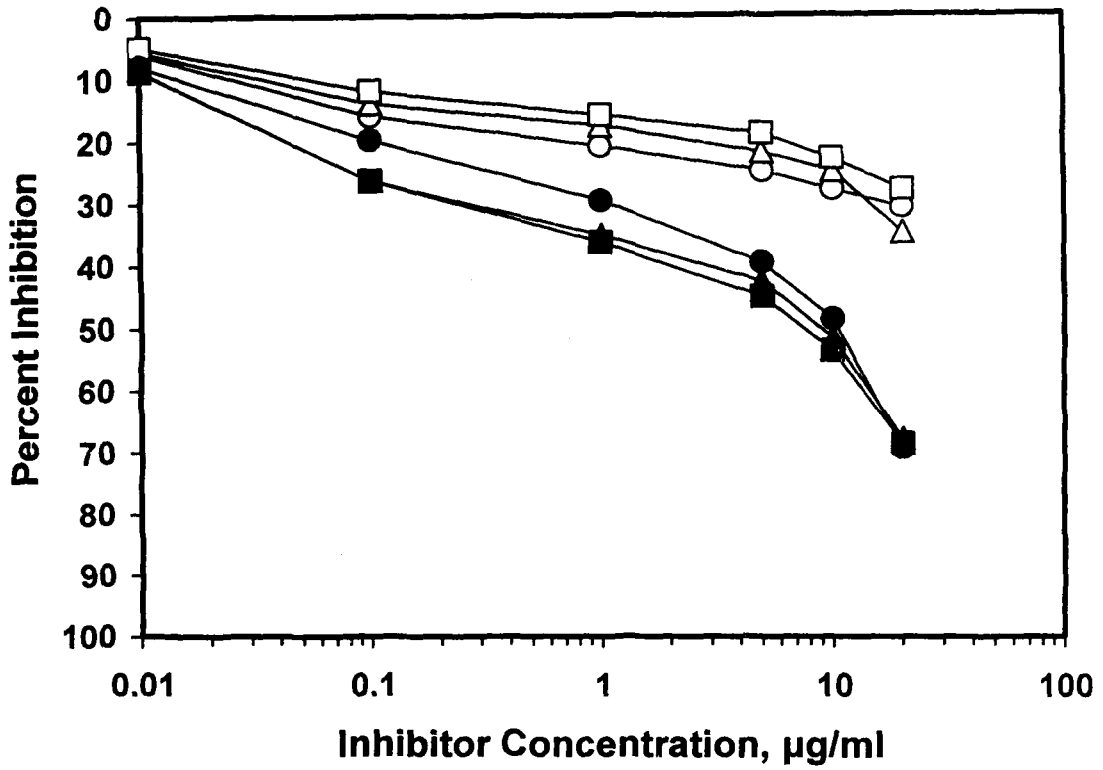


Fig. 60 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml). The above plot shows the inhibition in IgG isolated from serum samples 25, 31 and 32.

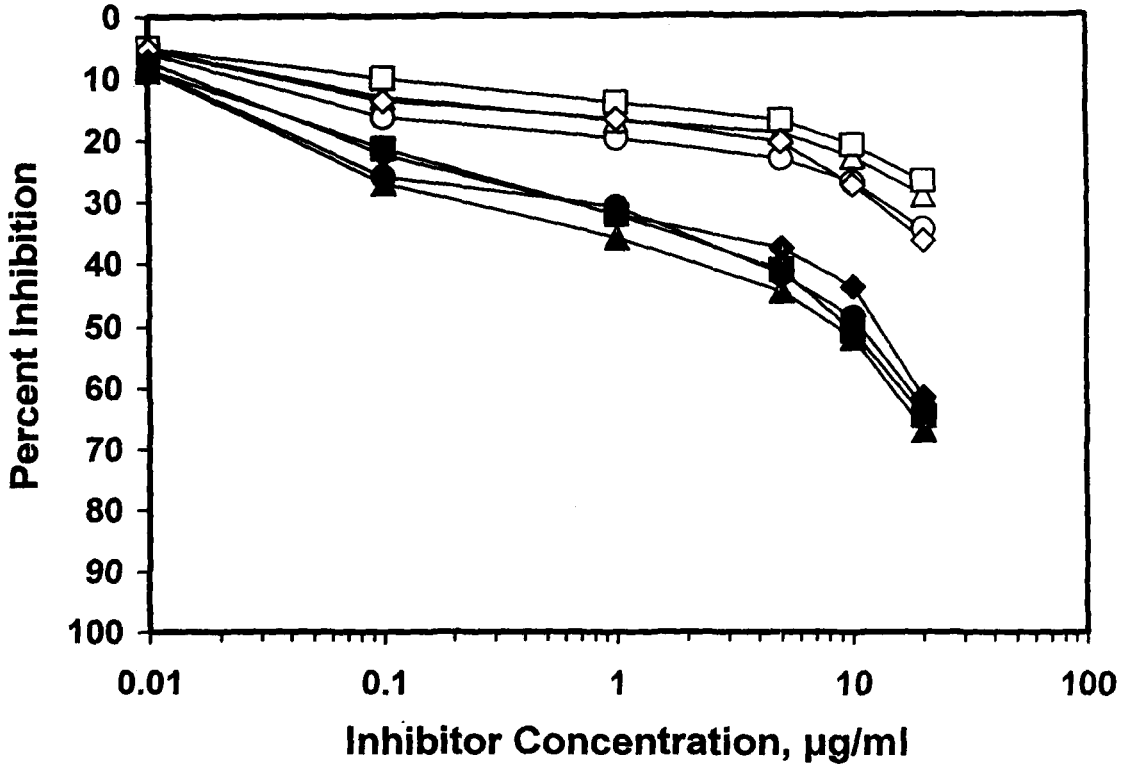


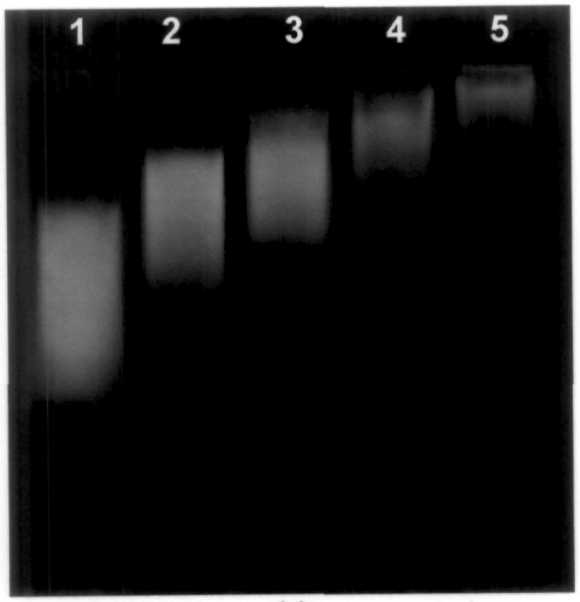
Fig. 61 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □, ◊) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■, ♦). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml). The above plot shows the inhibition in IgG isolated from serum samples 34, 37, 38 and 39.

TABLE 8

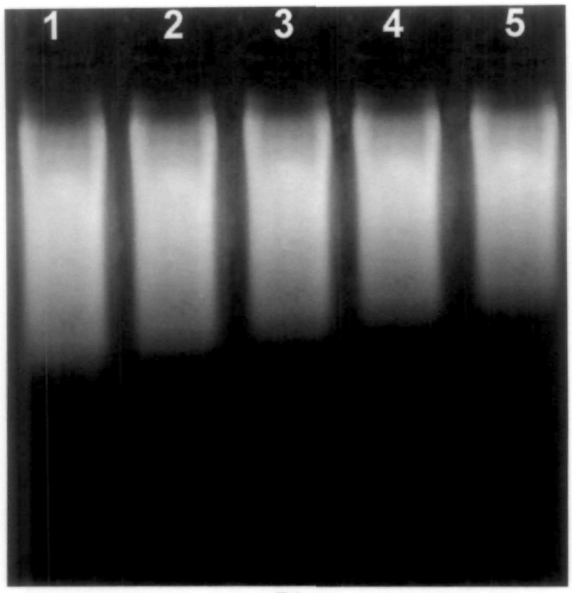
Competitive inhibition data of IgG isolated from type I diabetes patients

Sera no	Maximum percent inhibition at 20 μg/ml	
	Native human DNA	MG-Lys-Cu^{2+} modified human DNA
03	28.0	62.1
07	29.6	60.7
08	31.7	65.1
14	33.8	68.0
15	30.1	61.9
17	28.0	69.0
18	33.8	64.1
21	35.6	65.8
24	31.0	68.1
25	31.0	69.6
31	35.0	68.1
32	28.0	68.9
34	34.8	63.5
37	29.3	67.3
38	27.0	65.0
39	36.7	61.9
Mean ± SD	31.46±3.2%	65.56±7.1%

The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5μg/ml).



(a)



(b)

Fig. 62 Band shift of IgG from diabetic patient using (a) native and (b) MG-Lys-Cu^{2+} modified human DNA. Varying concentrations of IgG were incubated with a constant amount of DNA (0.5 μg) for 2 hr at 37 ^{0}C and overnight at 4 ^{0}C. Electrophoresis was carried out on 0.8% agarose gel for 2 hr at 30mA. Lane 1 contains native (or modified) human DNA while lanes 2-5 contain native or MG-Lys-Cu^{2+} modified human DNA with 20, 40, 60 and 80 μg of IgG from type I diabetes patient.

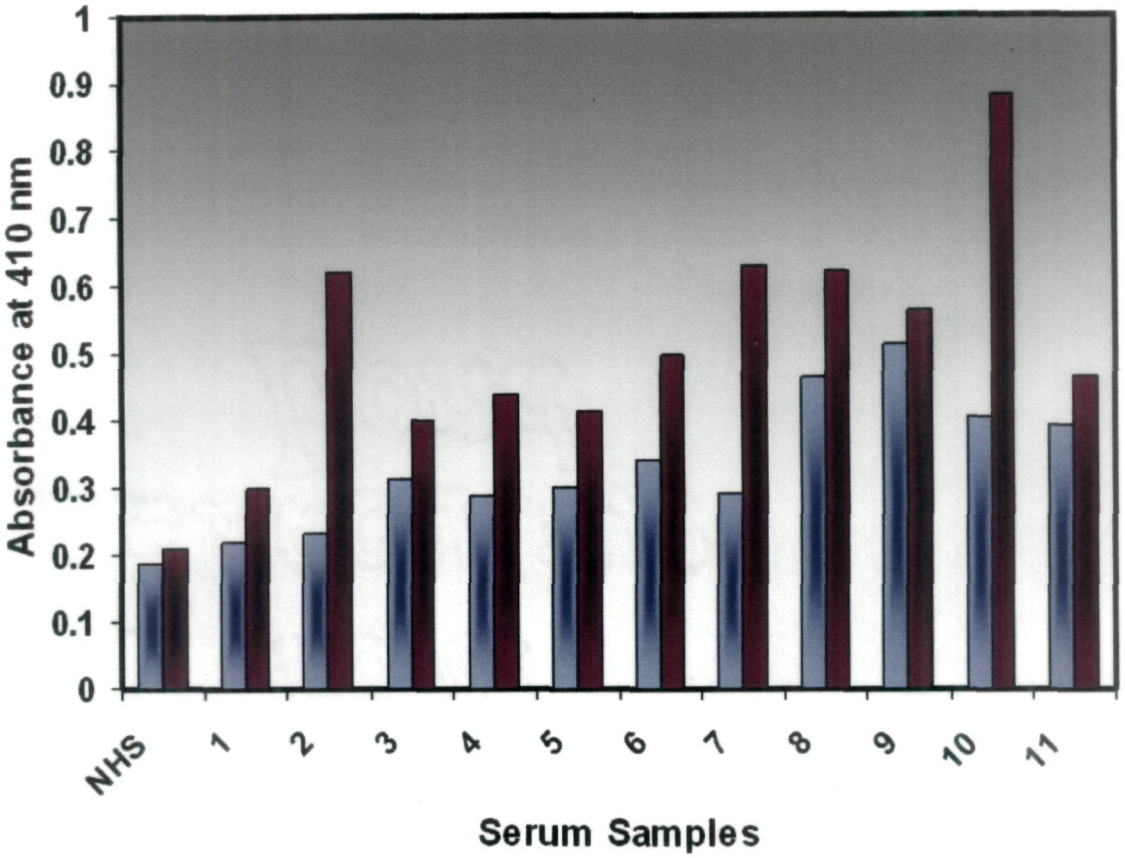


Fig. 63 Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.1-11) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

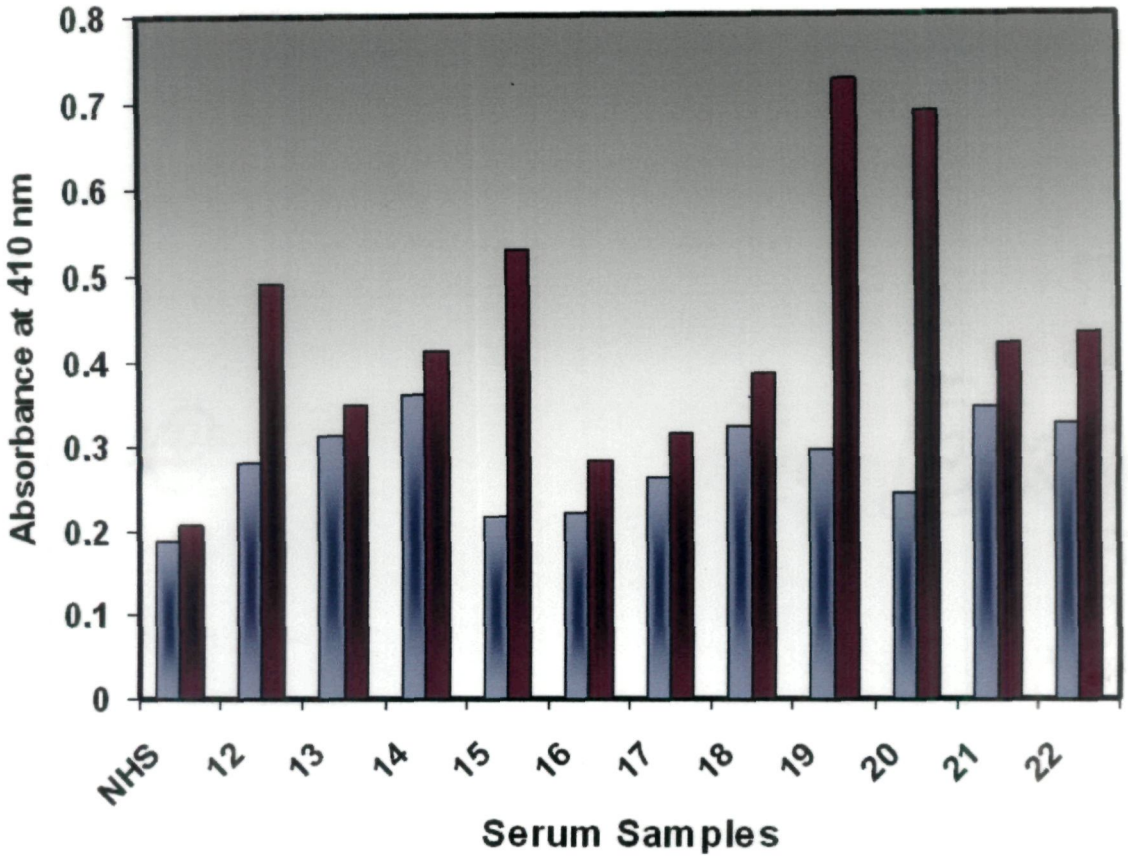


Fig. 64 Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.12-22) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).

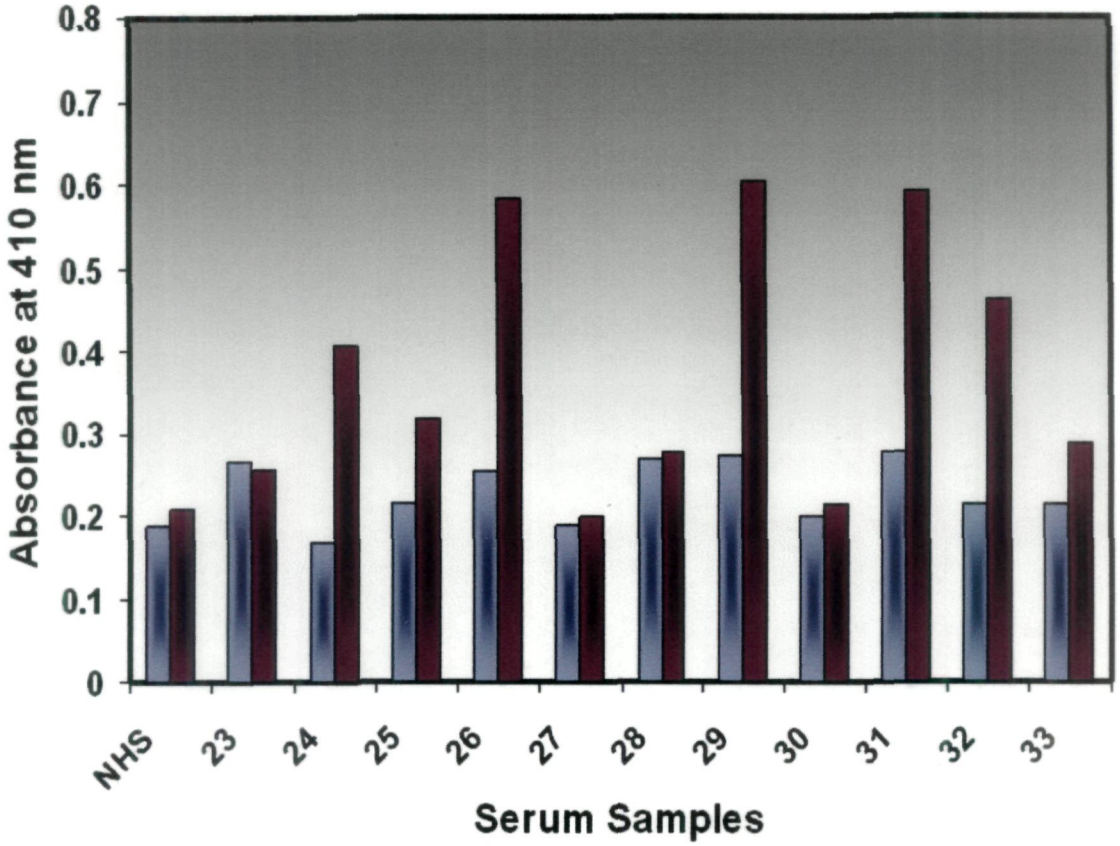


Fig. 65 Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.23-33) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).

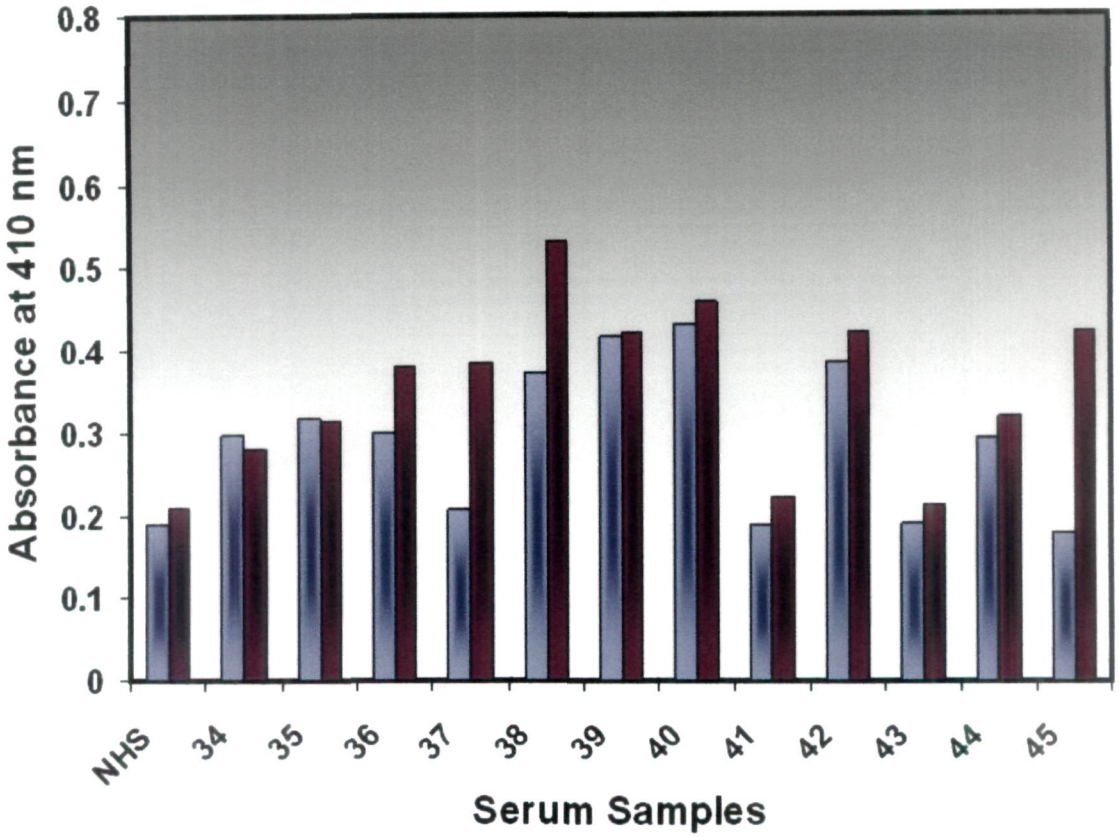


Fig. 66 Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.34-45) to native (■) and MG-Lys-Cu^{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).

antibodies to native and MG-Lys-Cu^{2+} modified human DNA was studied by competition inhibition solid phase assay. An inhibition in the range of 18% to 30.1% and 34.4% to 53% (Figs. 67-71) was recorded with native and MG-Lys-Cu^{2+} modified human DNA respectively. Mean inhibition for the entire sample tested with native human DNA was 23.9±3.0%, while for MG-Lys-Cu^{2+} modified human DNA, it was 44.6±5.3%. The inhibition studies results have been summarized in Table 9.

Purification of IgG from the sera of type II diabetes patient

IgG was isolated from the selected high binding serum samples of type II diabetes patients on a protein A-agarose column. IgG purified by affinity chromatography eluted as a symmetrical single peak (Fig. 72) and migrated as a single homogeneous band on SDS-PAGE under non-reducing conditions (Fig. 72 inset).

Binding of IgG from diabetes type II patients to native and MG-Lys-Cu^{2+} modified human DNA

Purified IgG from type II diabetes patients, were subjected to direct binding ELISA, on a microtitre plate coated with native human DNA and MG-Lys-Cu^{2+} modified human DNA to evaluate the amount required for antigen saturation. The saturation for modified human DNA was obtained at 80 μg/ml of IgG, while for native human antigenic saturation could not be ascertained because of its negligible binding. Therefore, for type II diabetes, IgG concentration was kept constant (80 μg/ml) in all further experiments.

The specific binding of the IgG isolated from type II diabetes patients was ascertained in competitive inhibition ELISA wherein an inhibition in the range of 24% to 36% and 49% to 57% was recorded with native and MG-Lys-Cu^{2+} modified human DNA respectively (Figs. 73-75). Mean inhibition for the entire sample tested with native human DNA was computed to be 29.3±3.6%, while for MG-Lys-Cu^{2+} modified human DNA, it was 53±7.3%. Table 10 summarizes the inhibition data of isolated IgG of diabetes type II group.

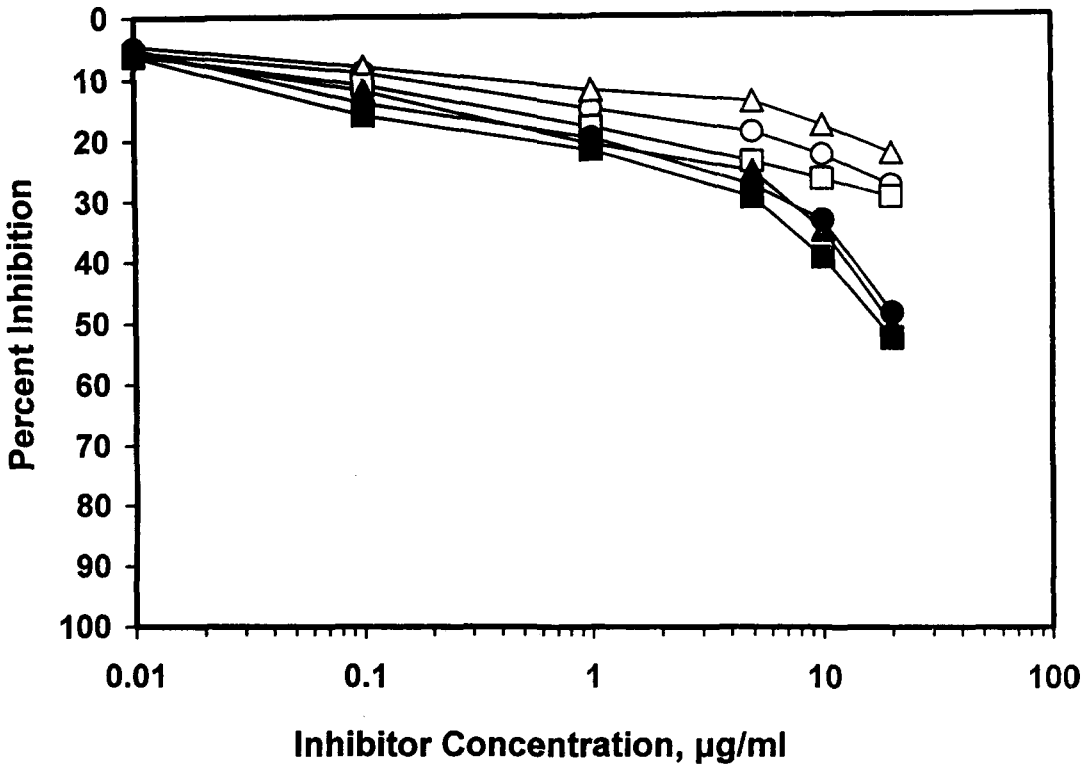


Fig. 67 Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 2, 7 and 8 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

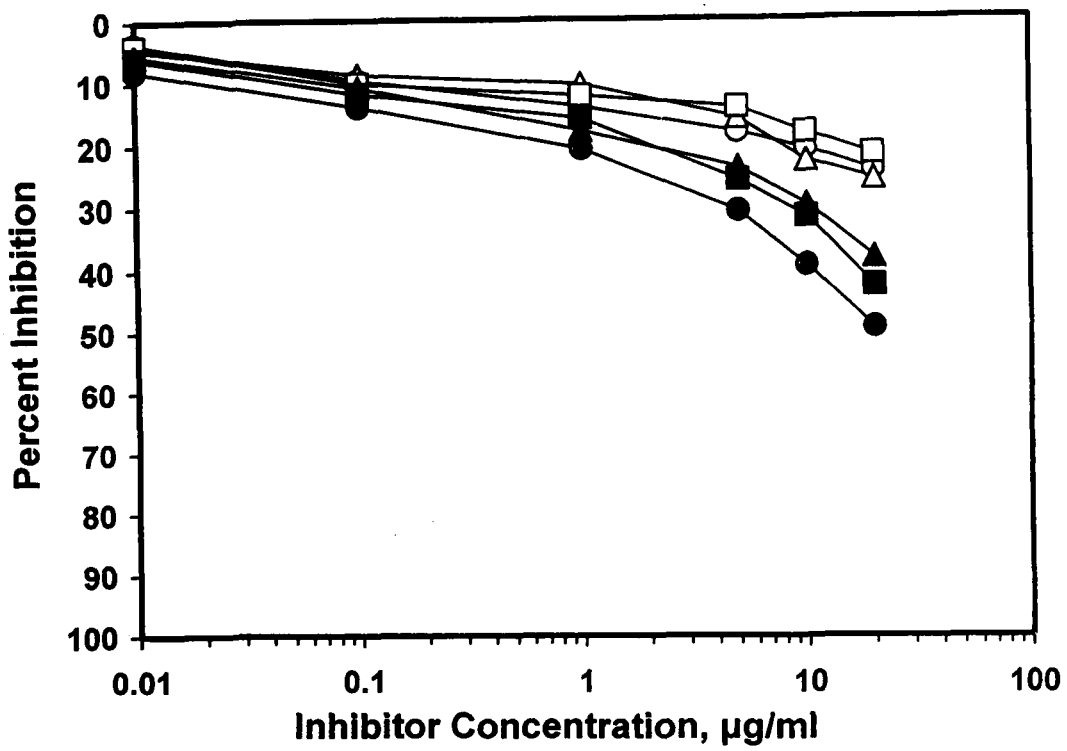


Fig. 68 Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 19, 12 and 15 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

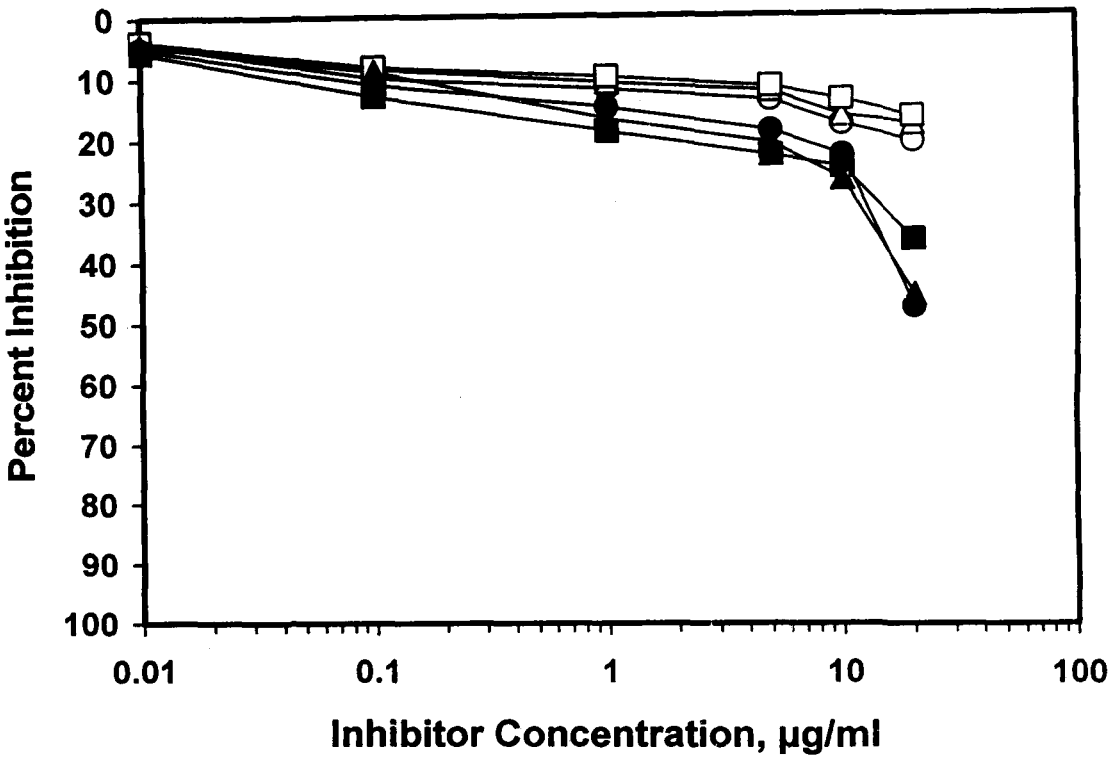


Fig. 69 Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (●, ▲, ■). Sera 19, 20 and 24 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

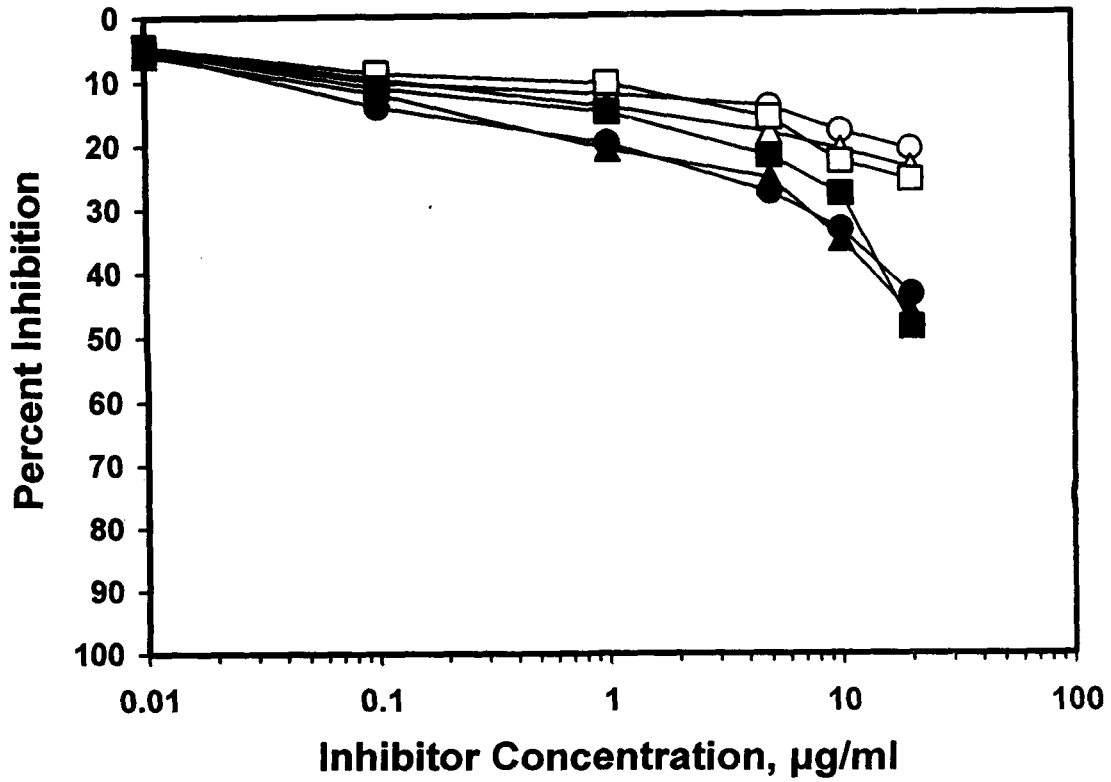


Fig. 70 Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). Sera 26, 29 and 31 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

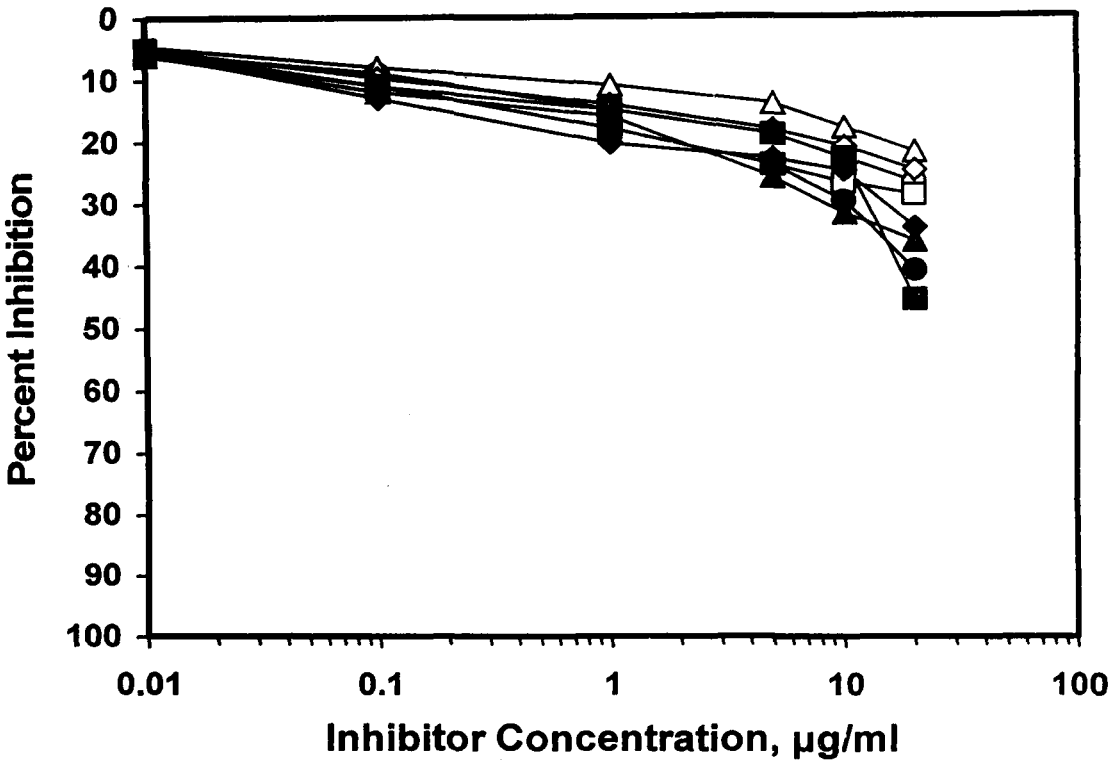


Fig. 71 Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □, ◊) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■, ♦). Sera 32, 37, 38 and 45 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

TABLE 9

Competitive inhibition data of serum autoantibodies in type II diabetes patients

	Maximum percent inhibition at 20 μg/ml	
Sera no	Native human DNA	MG-Lys-Cu^{2+} modified human DNA
02	28.2	49.0
07	22.8	51.0
08	30.1	53.0
10	24.6	50.0
12	26.0	38.6
15	22.0	43.2
19	20.9	48.0
20	18.0	46.0
24	16.8	37.0
26	21.0	44.0
29	24.0	47.0
31	26.0	49.0
32	27.0	41.3
37	22.0	36.5
38	29.0	46.0
45	25.0	34.4
Mean ± SD	23.9±3.0%	44.62±5.3%

The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5μg/ml).

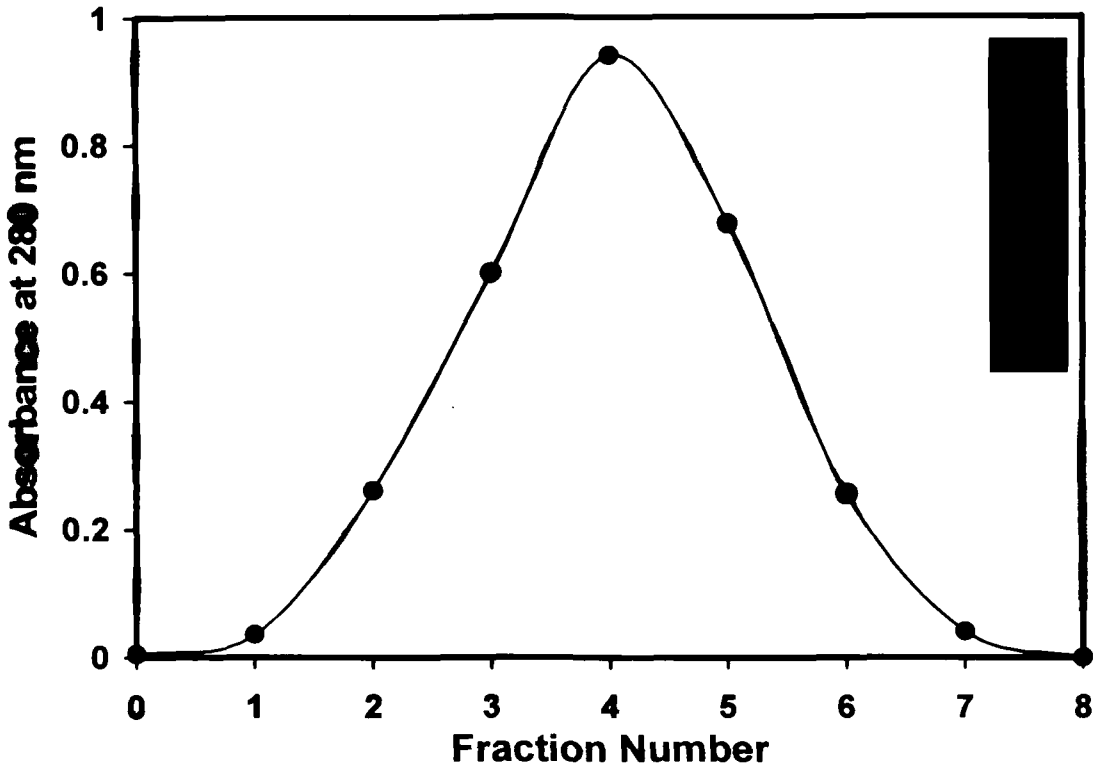


Fig. 72 Elution profile of IgG from the serum of diabetes type II patients on Protein A-agarose affinity column. Inset: SDS- PAGE of purified IgG on 7.5% polyacrylamide gel.

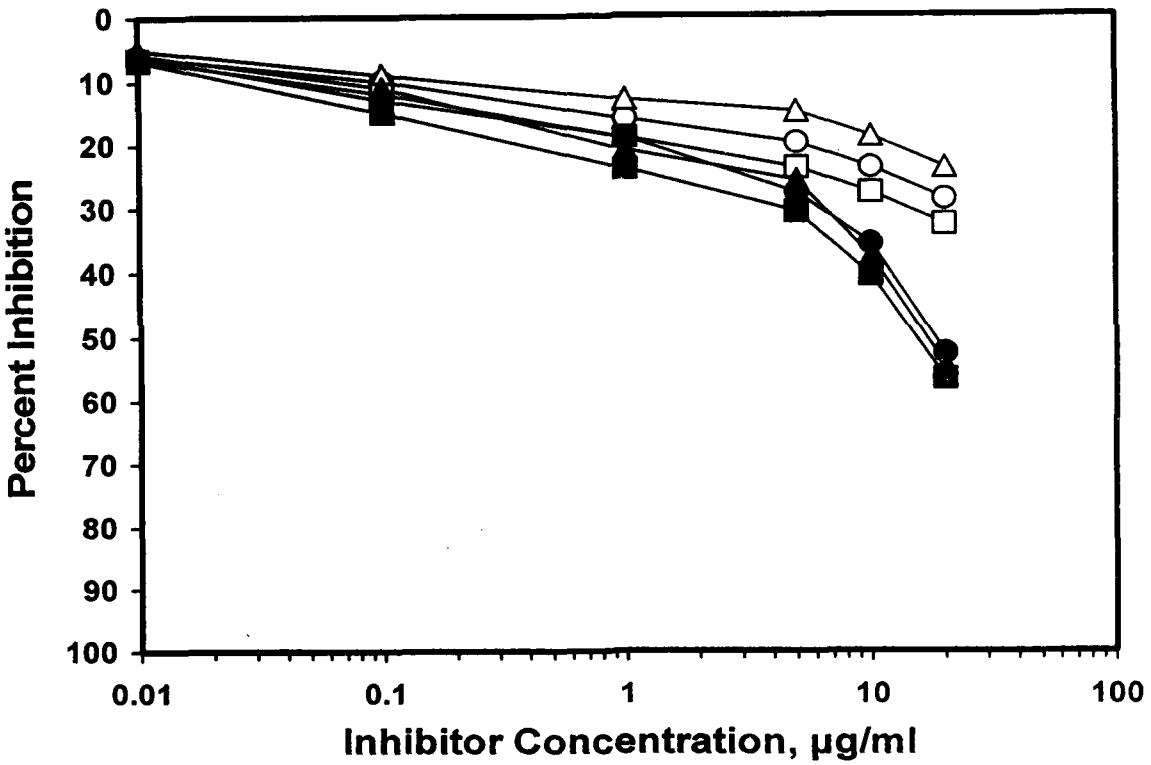


Fig. 73 Inhibition of IgG isolated from type II diabetes patients (samples 2, 7 and 8) by native (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (●, ▲, ■). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

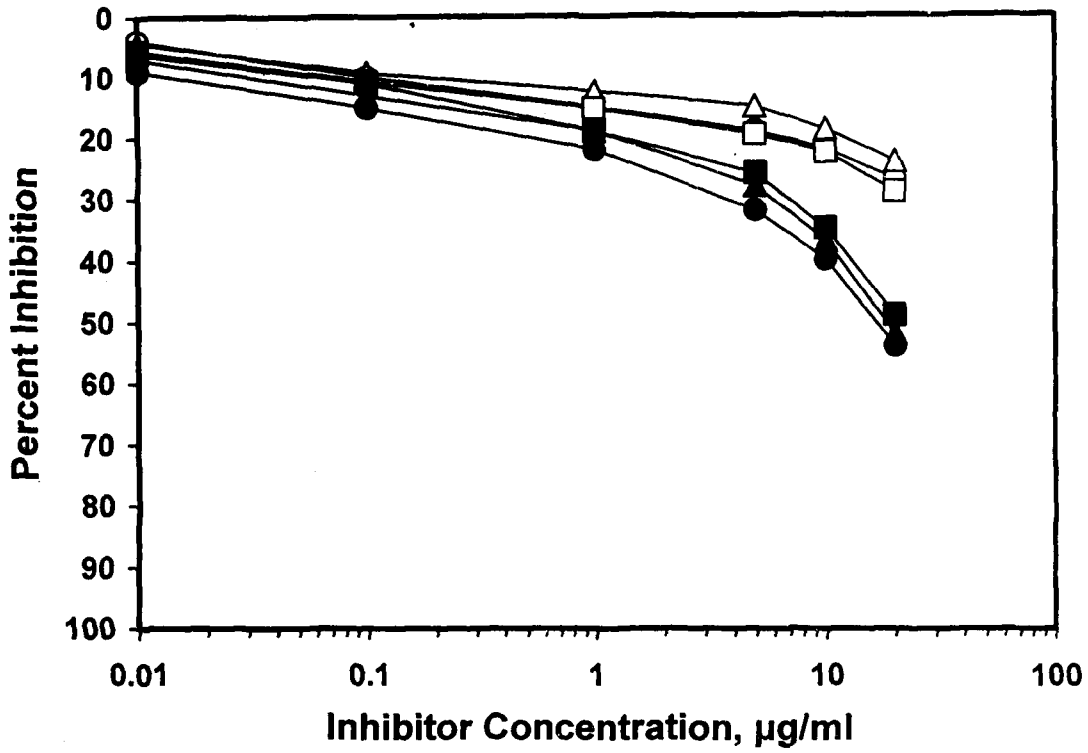


Fig. 74 Inhibition of IgG isolated from type II diabetes patients (samples 10, 19 and 29) by native (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (•, ▲, ■). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

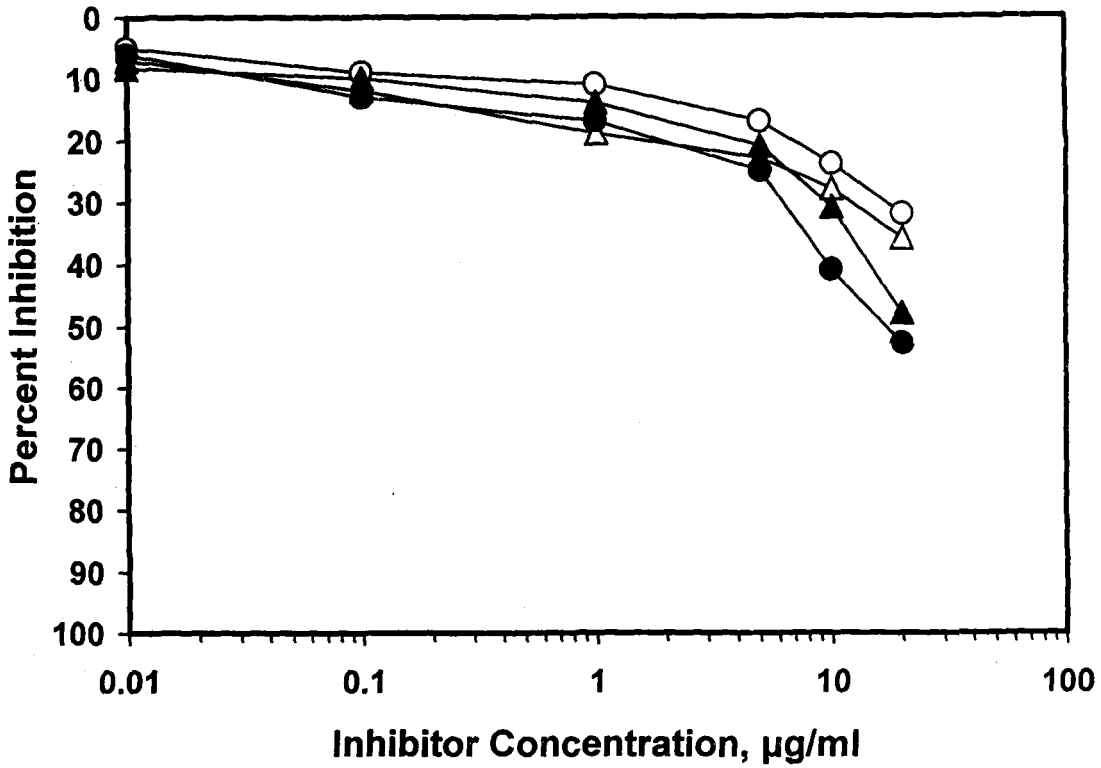


Fig. 75 Inhibition of IgG isolated from type II diabetes patients (samples 31 and 38) by native (o, Δ) and MG-Lysine-Cu^{2+} modified human DNA (•, ▲). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 µg/ml).

TABLE 10

Competitive inhibition data of IgG isolated from type II diabetes patients

	Maximum percent inhibition at 20 μg/ml	
Sera no	Native human DNA	MG-Lys-Cu^{2+} modified human DNA
02	29.0	53.0
07	24.0	55.0
08	33.0	57.0
10	27.0	54.0
19	25.0	52.0
29	29.0	51.0
31	32.0	53.0
38	36.0	49.0
Mean ± SD	29.3±3.6%	53±7.3%

The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5μg/ml).

Discussion

Endogenous MG is mainly formed spontaneously during glycolysis as a result of fragmentation and elimination of phosphate from glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Thornalley, 1996). Normally, 40-67% of MG is formed from G3P and 33-60% from DHAP, depending on the DHAP/G3P molar ratio (Phillips and Thornalley, 1993). G3P and DHAP can be converted to MG enzymatically (Ray and Ray 1981; Pompliano *et al.*, 1990) and nonenzymatically (Richard, 1991). Triosephosphate isomerase can convert both G3P and DHAP to MG. Triosephosphate isomerase is a very effective catalyst of isomerization and is present at very high cellular concentrations (Albery and Knowles 1976). Therefore, a significant concentration of cellular MG might be generated by triosephosphate isomerase even though its catalysis of degradation of G3P and DHAP to MG is very slow. DHAP can also be catalyzed to MG by methylglyoxal synthase in goat liver (Ray and Ray 1981). The instability of G3P at physiological pH was first reported in 1969 (Mel'nichenko *et al.*, 1969). This was followed by a study which found two reaction products, inorganic phosphate and MG, from nonenzymatic G3P reaction at pH 7.7, in the presence of increasing concentrations of the buffer catalyst lysine (Bonsignore *et al.*, 1973). Currently, it is accepted that the deprotonation of G3P or DHAP to an enediolate phosphate followed by the cleavage of phosphate group from the carbon skeleton results in the formation of MG (Richard, 1993). An estimated rate of nonenzymatic MG formation is 0.1 mmol/L/day (Richard, 1991). Under physiological conditions, minor sources of MG formation include metabolism of acetone from lipolysis and metabolism of threonine from protein catabolism (Casazza *et al.*, 1984; Lyles and Chalmers 1992). The conversion of acetone to MG involves two enzymes, acetone monooxygenase which converts acetone to acetol and acetol monooxygenase (AMO) which converts acetol to methylglyoxal. Semicarbazide-sensitive amine oxidase (SSAO) is a group of enzyme containing quinine and copper. Because SSAO contains a cofactor having carbonyls, it is sensitive to semicarbazide inhibition (Lyles, 1996). This enzyme is present in two forms, a soluble form in plasma and a membrane-bound form in tissues. Both forms can convert amino acetone to MG (Lyles, 1996). Increased SSAO activities are associated with diabetic complications, vascular disorders and heart disease. Under physiological conditions vascular endothelial cells are the major source of circulating SSAO (Stolen *et al.*, 2004).

Exogenous sources of MG include many food products, beverages (alcohol, tea, soft drinks, coffee etc.) (Nemet *et al.*, 2006). During food processing and storage, MG is formed as a by-product. For example, MG level in honey is in the range of 0.4-5.4 mg/kg, which comes from sugar degradation during the heating processes applied in manufacturing and storage (Nemet *et al.*, 2006). MG formation was also observed during the heating of glucose, fructose and maltulose. There are different amounts of MG in dairy products and alcoholic drinks coming from fermentation. In addition, cigarette smoking is also an exogenous source of MG. Gas chromatographic measurement revealed MG levels of 13.5-59.6 μg/cigarette (Fujioka and Shibamoto 2006). MG is found in plants, too. It has been reported that MG concentration is 30-75 micromolar in various plant species and it can be increased up to 6-fold by stress conditions such as salinity, drought and cold (Yadav *et al.*, 2005).

MG is mainly degraded to D-lactate via glyoxalase system. The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II and a cofactor, reduced glutathione (GSH) (Racker, 1951).

Apart from glyoxalase system, the non-glyoxalase metabolism of MG also exists. For example, (a) Aldose reductase catalyzes the conversion of MG to acetol (95%) and D-lactaldehyde (5%), with NADPH as a cofactor (Vander *et al.*, 1992). (b) MG reductase catalyzes the reduction of MG to L-lactaldehyde (Ting *et al.*, 1965). (c) MG dehydrogenase catalyzes the oxidation of MG to pyruvate (Ray and Ray, 1982).

MG is often found at high levels in the blood of diabetic patients, where it covalently depletes glutathione (GSH), leading to serious toxicological effects. In comparison to the parent sugar, MG is more likely to cross-link with the amino groups of various proteins, forming stable end products called advanced glycation end products (AGEs) (Bourajjaj *et al.*, 2003). In addition, MG may modify cross-linked lysine and arginine residues, leading to alterations in the protein characteristics (Vander *et al.*, 1992). The reaction of proteins with MG has been shown to produce reactive oxygen species (ROS) such as O_2 during the glycation reaction (Yim *et al.*, 1995); ROS and oxidative stress can damage many biological molecules (e.g., DNA) and cause various cell injuries. Studies have shown that oxidative stress and AGE formation are associated with impaired cognitive processes in diabetic patients

(Messier and Gagnon, 1996; Kyriaki, 2003), suggesting that this may be linked to MG toxicity.

The consumption of 2–3 cups of coffee per day can lead to a daily MG intake of up to 1 mg (Kasai *et al.*, 1982). The concentration of MG in blood samples from normal controls was about 1 μM whereas this level was increased 5-6 fold in blood samples of diabetes patients with insulin dependent diabetes mellitus and 2-3 fold in blood samples of non-insulin-dependent diabetes mellitus (McLellan *et al.*, 1994), and may persist for several months or years in diabetic patients. The concentration of MG in human lenses is normally about 20 times higher than in plasma (Phillips and Thornalley, 1993). It has been shown that physiological levels of MG can induce DNA cleavage and ROS generation and decrease cellular adhesion in mononuclear cells (Chan and Wu, 2006). A higher concentration can be more deleterious.

Methylglyoxal is well known to modify many different compounds in the cell, including free amino acids (Lo *et al.*, 1994; Takahashi, 1977) proteins (Lo *et al.*, 1994; Westwood *et al.*, 1994) and nucleic acids (Krymkiewicz, 1973). It has been shown that methylglyoxal reacts with guanine residues in DNA to form a tricyclic compound (Shapiro *et al.*, 1969, Vaca *et al.*, 1994). Thus, methylglyoxal may induce mutations by reacting with guanine residues and by the subsequent formation of mispairs. Indeed, methylglyoxal is mutagenic in *Salmonella typhimurium* strains containing the pKM101 plasmid, *Escherichia coli* WP2 *uvrA* and WP2 *uvrA* pKM101, and *Saccharomyces cerevisiae* strain D7 (Bronzetti *et al.*, 1987). In cultured CHO cells, MG induced sister chromatid exchanges, single-stranded DNA breaks and DNA–protein crosslinks have been reported (Brambilla *et al.*, 1985).

Diabetes affects more than 180 million people worldwide (World Health Organization, Diabetes Fact Sheet, 2006). About 5–10% of these cases are Type I diabetes (American Diabetes Association, 2007). T1DM usually results from the body's failure to produce insulin. This leads to hyperglycemia, and insulin therapy is required to control blood glucose levels. Glucose forms reversible early glycation products with proteins and DNA. Glycated hemoglobin ((HbA1C) is used to assess long-term glycemic control in diabetes. Early glycation products may undergo further rearrangement and oxidation to produce advanced glycation end products (AGEs)

which alters the structure of proteins and DNA irreversibly (Thornalley *et al.*, 1999). Reactive carbonyl species (RCS) such as methylglyoxal and glyoxal can also form AGEs. In fact, RCS are 20,000 times more reactive than glucose in glycation processes (Thornalley, 2005). In diabetes mellitus, RCS may arise from various sources. Methylglyoxal is formed through enzymatic and non-enzymatic degradation of glucose, and thus in hyperglycemic conditions they may be produced in excess (Phillips and Thornalley, 1993; Wells-Knecht *et al.*, 1995; Thornalley *et al.*, 1999; Beisswenger *et al.*, 2001). Altered glucose metabolism may result in an increased formation of methylglyoxal via the polyol pathway (Phillips and Thornalley, 1993; Chang *et al.*, 2002). Increased protein and lipid catabolism may also be seen in poorly controlled diabetes producing elevated levels of aminoacetone and acetone, which are precursors of methylglyoxal (Kalapos, 1999). Methylglyoxal form AGEs by binding with free sulfhydryl and amino groups of proteins (Thornalley, 2003; Zeng and Davies, 2005), resulting in endothelial dysfunction (Rodriguez-Manas *et al.*, 2003; Wautier and Schmidt, 2004) and oxidative stress (Kislinger *et al.*, 2001; Scivittaro *et al.*, 2000; Wu, 2005). There is a growing body of evidence that methylglyoxal- and glyoxal-derived AGEs are involved in the complications of diabetes including nephropathy, neuropathy and retinopathy (Hammes *et al.*, 1999; Misselwitz *et al.*, 2002; Lieuw-A-Fa *et al.*, 2004; Hwang *et al.*, 2005).

Sugars and other reactive carbonyl compounds bind spontaneously to nucleophilic amino groups of amino acids and proteins in a nonenzymatic process (glycation) (Maillard, 1912). It is well established that proteins are readily glycated *in vivo*. The first glycation product to be detected *in vivo* was hemoglobin (Hb) A1c, the Amadori product of Hb A (Koening *et al.*, 1977). *In vivo*, early glycation products, such as the amadori product, are further converted into the heterogeneous group of advanced glycation end-products (AGEs). AGEs accumulate on serum proteins and in various tissues, particularly during aging, diabetes and renal failure (Vlassara *et al.*, 1994). Elevated AGE levels contribute to the development of diabetic and uremic complications, such as atherosclerosis (Park *et al.*, 1998), nephropathy and retinopathy (Singh *et al.*, 2001). In analogous reactions, glycation may also affect DNA. *In vitro*, nucleobases and dsDNA react with sugars in a similar way as proteins (Lee and Cerami, 1987; Knerr and Severin, 1993; Singh *et al.*, 2001). The exocyclic amino group of 2□-deoxyguanosine is particularly prone to glycation reactions,

leading to the formation of N^2-carboxyethyl, N^2-carboxymethyl, N^2-(1-carboxy-3-hydroxypropyl), and N^2-(1-carboxy-3,4,5-trihydroxypentyl) modifications, as well as cyclic dicarbonyl adducts (Knerr & Severin 1993; Ochs & Severin 1994; Larisch *et al.*, 1998; Seidel & Pischetsrieder, 1998). The two diastereomers of N^2-carboxyethyl-2□-deoxyguanosine ($CEdG_{A,B}$) are stable reaction products that are formed from a variety of glycating agents, such as glucose, ascorbic acid, glyceraldehyde, dihydroxyacetone (DHA), or methylglyoxal (Larisch *et al.*, 1998; Seidel & Pischetsrieder 1998; Frischmann *et al.*, 2005). Recently, carboxyethylated nucleobases were detected in human urine (Schneider *et al.*, 2004), indicating the formation of DNA-AGEs in healthy humans. A significantly increased number of CEdG positive cells were immunostained in glomeruli of patients with diabetic nephropathy as compared to healthy controls (Li *et al.*, 2006), as well as in glomeruli of diabetic rats (Nakamura *et al.*, 2007). DNA AGEs are potentially genotoxic compounds because they induce depurination (Seidel & Pischetsrieder, 1998) as well as single strand breaks and lead to mutations (Pischetsrieder *et al.*, 1999) *in vitro*.

The reaction of methylglyoxal with amino acids generates crosslinked methylglyoxal dialkylimine cation radical, the enediol anion radical of methylglyoxal and the superoxide ion. A direct 1-electron transfer between a Schiff base methylglyoxal dialkylimine (or its protonated form) and methylglyoxal is responsible for the generation of the cross-linked radical cation and the radical counter anion of methylglyoxal. Under aerobic conditions, molecular oxygen can then accept an electron from the methylglyoxal anion to generate the superoxide ion. The formation of methylglyoxal dialkylimine radical cation and the enediol radical anion of methylglyoxal are independent of molecular oxygen or metal ions. The later stage needs the presence of molecular oxygen. The formation of α-ketoaldehydes during glycation is a critical step that leads to protein cross-linking and formation of radical cation sites on the cross-linked proteins. The counter anions, superoxide and hydrogen peroxide generated from MG anion can initiate free radical chain reactions leading to damage of biomacromolecules in close proximity to reaction sites. (Suji and Sivakami, 2007)

In the present study, human placental DNA was modified by 40 mM methylglyoxal (MG) and 40 mM lysine in the presence of 300 μM (Cu^{2+}) copper

sulphate. This reaction system (MG-Lys-Cu^{2+}) caused extensive damage to the human DNA, leading to the formation of single strand breaks and base modifications. The MG-Lys and MG-Lys-Cu system gave 67% and 76% hyperchromicity respectively compared to native double stranded analogue. However, when MG, lysine and Cu^{2+} were individually incubated with human DNA under identical conditions, no significant change in hyperchromicity was found. Furthermore, MG-Cu^{2+} and Lys-Cu^{2+} systems also did not show any detectable change in UV-spectral profile. The change in hyperchromicity of MG-Lys and MG-Lys-Cu^{2+} system could be attributed to the single strand breaks, destabilization of hydrogen bonds, and modification of nitrogenous bases which result in the destruction of chromophoric groups through attack on the sugar-phosphate back bone. Furthermore, an interesting detail is the new additional peak found at about 330 nm, when human DNA was incubated with MG-Lys and MG-Lys-Cu^{2+} systems. This could be attributed to the generation of DNA-AGEs. MG-Lys-Cu^{2+} derived DNA-AGEs show the highest absorbance, suggesting Cu^{2+} enhances AGEs formation. A study by Schmitt (Schmitt *et al.*, 2005) strongly supports our results where they found an extra peak in the same range of 320-330 nm when HSA was incubated with MG, suggesting the formation of glycation induced AGEs. From the UV absorbance data it can be concluded that absorbance between 320 and 370 nm (Schmitt *et al.*, 2005) can be used to judge the onset of AGE formation of DNA that are derived from the reaction with modifier (MG-Lys-Cu^{2+}). Moreover, amino acids (lysine and arginine) treated with MG have been reported to give electron paramagnetic resonance signals (Pethig and Szent-Gyorgyi, 1977), indicative of the formation of free radicals ($^{\bullet}OH$ and $O_2^{\bullet-}$). The formation of free radicals ($^{\bullet}OH$ and $O_2^{\bullet-}$) in this study, was confirmed by quenching studies including superoxide dismutase (specific quencher for $O_2^{\bullet-}$) and mannitol ($^{\bullet}OH$ radical quencher). The results presented here, therefore, indicate that the glycation reaction of MG with lysine in the presence of Cu^{2+} may lead to oxidative damage of DNA through a mechanism that involves hydroxyl radicals.

Hydroxyl radical production, expressed as TBARS nmole/ml, was found to increase as a function of MG and lysine. Addition of metal ions further increased the production of TBARS. MG-Lys and MG-Lys-Cu systems generated 13 and 24 nmol TBARS ml^{-1} respectively. However, radical scavenger, catalase, and copper chelator

significantly inhibited the generation of hydroxyl radicals by the MG–lys–Cu^{2+} system. The result suggests that redox reactions of copper may be facilitating the generation of hydroxyl radical by the reaction of MG with lysine.

Comet assay is well established as a sensitive method for detecting single or double strand breaks, alkali labile sites, abasic sites and relaxed chromatin formation in the DNA of single cells. The resulting DNA fragments move ahead of the intact nuclear DNA forming a comet-like structure as smaller fragments move faster than the larger fragments and the intact DNA. Therefore, comet assay can be used to test the genotoxicity of AGEs in cultured cells including freshly isolated human lymphocytes. Using the comet-assay as an endpoint for DNA damage, the study shows that MG-Lys and MG-Lys-Cu^{2+} treated human lymphocytes were genotoxic in human lymphocytes. However, MG and lysine alone did not cause any significant change in lymphocyte DNA. This is evident from the DNA breakage and subsequent formation of the comet tail. The results clearly establish that MG-Lys system is capable of DNA breakage in lymphocytes which is due to the formation of distinct tail from the diffused head. However, in the presence of Cu^{2+} the damage of lymphocyte DNA was found to be enhanced by 36% suggesting Cu^{2+} enhances the genotoxicity of MG+Lys system by increasing free radical ($^{\cdot}OH$) generation, as discussed before. Furthermore, in MG-Lys-Cu treated lymphocytes, an increase in OTM, % DNA in tail and tail length was found to be 316%, 246% and 92% respectively when compared with untreated lymphocytes.

Previously, it was shown that an *in vitro* AGE-modified plasmid transformed into *Escherichia coli* resulted in an increased mutation frequency (Pischetsrieder *et al.*, 1999). This yields the idea that glycating agents may directly exert modification of DNA and thus induce DNA-damaging or mutagenic effects.

Appreciable evidence for generation of strand breaks (single and double) in the human DNA as a consequence of MG-Lys-Cu^{2+} modification was gathered by agarose gel electrophoresis. The electrophoretic pattern of MG-Lys-Cu^{2+} glycated human DNA revealed an increasing mobility with increasing incubation time. It was observed that at 24 hr incubation the mobility of glycated DNA was maximum and further incubation did not have any consequential effect on migration pattern. This suggests that maximum damage to DNA was at 24 hr incubation. It may be due to the

generation of single strand breaks by glycation induced intermediates or free radical generation ($^{\cdot}OH$) or both, which may result in the formation of small size DNA showing faster mobility compared to the native form.

Fluorescence spectra of both native and MG-Lys-Cu glycated human DNA was recorded in the wavelength range of 380-500 nm. Generation of fluorogenic AGEs in glycated-DNA samples was measured using excitation wavelength of 370 nm (λ_{ex}) and emission wavelength of 450 nm (λ_{em}) (Minorova *et al.*, 2005). Under identical conditions, DNA alone does not give any fluorescence. Glycation of DNA by methylglyoxal and lysine generated fluorescent DNA-AGEs characterized by emission maxima of 450 nm. The fluorescence intensity increased upon incubation with copper sulphate suggesting that copper enhances the formation of DNA- AGEs.

Earlier studies have demonstrated that the structural alteration in DNA following damage by various agents result in the generation of single strands in the DNA molecule (Shishido and Ando, 1974; Yamasaki *et al.*, 1997). Native and modified DNA was subjected to digestion by nuclease S1, in order to confirm the generation of single strand breaks. The results show substantial digestion of modified DNA by nuclease S1, while native DNA remained unaffected. These observations clearly demonstrate that sufficient distortions are caused in DNA helix by MG-Lys-Cu^{2+} system, rendering it susceptible to digestions by single strand specific nuclease S1.

The CD spectrum of modified human DNA shows a decrease of 27.56 % in the positive ellipticity at 275 nm as compared to native form. The decrease in ellipticity with slight change in the peaks of native DNA after methylglyoxal modification may be due to unstacking of bases as a result of helix destabilization. The structural perturbations suggest unfolding of DNA which might be a result of single strand breaks.

Stacking of bases and hydrogen bonding stabilize DNA structure and their disruption causes denaturation (Casperson and Voss, 1983; Thomas, 1993). The native human DNA, upon MG-Lys-Cu^{2+} modification, showed susceptibility to temperature when subjected to thermal denaturation studies. The melting temperature of the modified human DNA was found to be 10 ^{o}C less as compared to the melting

temperature of native human DNA, used as reference. This could be attributed to the presence of single strand regions in the modified DNA and also a partial destruction in secondary structure. The decrease in Tm, therefore, points towards the destabilization of base stacking and hydrogen bonding and consequent helix disruption.

Several reports (Brownlee *et al.*, 1984; Mullarkey *et al.*, 1990; Bucala *et al.*, 1991; Simpson *et al.*, 1992) have shown that glycated protein generated superoxide anions, which initiated lipid peroxidation. During incubation of MG with lysine, the formation of superoxide anion was gradually increased in a time-dependent manner. It has also been reported that the incubation of threose with *N*-acetyl-lysine produced 16.2 nmol $O_2^{\bullet -}ml^{-1}h^{-1}$ (Ortwerth *et al.*, 1998). In this study, the incubation of MG with lysine produced 28.2 nmol $O_2^{\bullet -}$ $ml^{-1}h^{-1}$. The result suggests that DNA strand breakage by MG–Lys–Cu^{2+} system may be dependent on the formation of superoxide anion in this system. However, MG and lysine alone generated only 2.46 and 1.96 nmol $O_2^{\bullet -}$ $ml^{-1}h^{-1}$ respectively. The production of superoxide radical was gradually inhibited by increasing concentration of superoxide dismutase, further confirming the formation of superoxide radical.

Glycation adducts of DNA may have potential as biomarkers since all nucleated cells contain the same DNA content and should reflect the relative level of MG in the target tissue. Reaction of double-stranded DNA with MG or glucose *in vitro* produces primarily N^2-carboxyethyl-2′-deoxyguanosine (CEdG), suggesting to be the likely major adduct formed *in vivo* (Papoulis *et al.*, 1995; Frischmann *et al.*, 2005). This implies that CEdG might be a useful biomarker for monitoring oxoaldehyde-induced stress in response to enhanced glycolytic flux or environmental exposure to MG. The preparative HPLC was employed for the synthesis of the standard, carboxyethyl deoxyguanosine (CEdG). The standard adduct, CEdG formed by preparative HPLC was further proved by nuclear magnetic resonance analysis. The retention time of the synthesized CEdG was found to be 14.399 min. The HPLC performed for native and glycated DNA showed remarkable difference in peak retention times. The extra peak at a retention time of 14.249 min in modified DNA is characteristic of N^2-(1-Carboxyethyl)-2-deoxyguanosine (CEdG) adduct. This is in accordance with the standard CEdG results wherein also when deoxyguanosine was

exposed to dihydroxyacetone, a distinct peak at retention time of 14.399 min was observed. However, native DNA did not showed the peak at this retention time. The LC-MS technique was performed so as to detect the glycated adduct, CEdG formed with the double stranded human DNA. The acid hydrolysate of MG-Lys-Cu^{2+} glycated human DNA showed a m/z value of 338 in the negative ion mode. This is in conformity with the standard, CEdG wherein also we obtained the m/z value of 338, which is the characteristic of CEdG adduct formed with human DNA.

For a typical nonenzymatic glycation reaction to occur, the carbonyl group of a reducing sugar must first react with the free amino groups of a protein to form a Schiff base with the latter and the intermediate then undergoes an Amadori rearrangement to generate a stable ketoamine product. Recently, it has been reported that the reaction of deoxy-guanosine (dG) with MG proceeds via Amadori pathway (Li *et al.*, 2008). In our case the ESI-MS, mass-spectroscopic data has shown similar results, i.e., the reaction of human DNA with MG-Lys-Cu^{2+} proceeds via the classic Amadori pathway and yields glycation-like products similar to those generated between a nucleoside and a carbohydrate. This is in conformity with the results we obtained for ESI-MS. The ion at m/z 341, 679, 268 and 385 is consistent with a $[Schiff\ base+H]^+$, $[Schiff\ base+H]^+$ dimer product, dG-H_2O and a fragment formed by the degradation of MG reacting with the Schiff base product, or its enaminol or Amadori intermediate respectively.

Native double stranded DNA (B- conformation) *per se* is non immunogenic (Isenberg et al., 1994; Habib *et al.*, 2005), whereas upon modification with carcinogens, drugs, hormones, free radicals etc., DNA undergoes structural perturbations which lead to the generation of neo-epitopes on the molecule which are recognized as foreign by the immune system and hence induces the immune response. Immunization with single stranded DNA can elicit a limited antibody response, while double stranded RNA, RNA-DNA hybrids, DNA modified with carcinogens, drugs etc. or DNA complexes with DNA binding proteins are effective immunogens (Stollar, 1975; Anderson *et al.*, 1988; Desai *et al.*, 1993; Moinuddin and Ali, 1994; Dixit *et al*, 2005; Habib *et al.*, 2005; Khan *et al.*, 2006). In contrast to native mammalian DNA (B-form), bacterial DNA is a stronger immunogen (Krieg, 2002). Moreover, the initial immune response to DNA is dominated by lower affinity IgM

Ab primarily specific for single stranded (ss), denatured mammalian DNA. As the immune response progresses, the Ab isotype switches to IgG and the Ab acquires increasing affinity for native, double stranded (ds) DNA (Tillman *et al.*, 1992).

Antibodies against MG-Lys-Cu modified human DNA were induced in rabbits by immunizing with MG-Lys-Cu modified human DNA complexed with methylated bovine serum albumin. The MG-Lys-Cu modified human DNA was a potent immunizing stimulus, inducing high titre antibodies. Antigenic specificity of anti-MG-Lys-Cu modified human DNA IgG was ascertained by competitive binding assay. A maximum of 88.5% inhibition in the antibody activity was obtained at inhibitor (immunogen) concentration of 20 μg/ml and just 2.8 μg/ml of the inhibitor concentration caused 50% inhibition, clearly indicating very high specificity and affinity of the induced antibodies towards the immunogen, i.e. the MG-Lys-Cu modified human DNA. Low inhibition with native human DNA (35.1%) and moderate inhibition with MG-Lys-Cu modified calf thymus DNA (64.2%) and MG-Lys-Cu modified human lymphocyte DNA (72.5%) was obtained. This shows that majority of the antibodies are directed towards the MG-Lys-Cu modified epitopes on the DNA.

Visual detection of interaction between immune IgG and the immunogen was facilitated by the gel retardation assay. The result reflects high affinity of the induced antibodies for the immunogen. However, anti- MG-Lys-Cu- human DNA IgG did not show any appreciable binding with the native human DNA. These results further confirm that the induced antibodies are predominantly directed against the antigenic determinants generated as a result of MG-Lys-Cu modification of human DNA.

Native calf thymus and plasmid DNA showed inhibitions of 33.3% and 32.1% respectively, whereas their MG-Lys-Cu modified forms showed enhanced inhibition of 64.2% and 54.2% respectively. These results demonstrate the preferential recognition of MG-Lys-Cu modified epitopes by the immune IgG. However, ROS-modified form of calf thymus DNA and plasmid DNA inhibited antibody activity to a lesser extent possibly because the ROS modified epitopes are not so distinctly recognized by the induced antibodies. Amongst MG-Lys-Cu modified bases, guanine showed substantial inhibition (78%). Previous reports have demonstrated that guanine

or its nucleoside form (deoxyguanosine) is particularly susceptible to modification by reactive carbonyl species like methylglyoxal, glyoxal, glucose, etc. generating carboxyethyl deoxyguanosine (CEdG) as major adduct (Ochs and Severin, 1994; Papoulis *et al.*, 1995; Seidel and Pischetsrieder, 1998; Frischmann *et al.*, 2005). However, carboxyethyl guanosine (CEG), carboxymethyl deoxyguanosine (CMdG) and carboxymethyl guanosine (CMG) are also formed as minor adducts depending on the reaction conditions (Nissl *et al.*, 1996). All these adducts are due to the attack of RCS on guanine residue of the DNA.

Increased glycation and, in particular, accumulation of tissue and serum AGEs have an important role in the pathogenesis of diabetic complications. Studies in the past have focussed largely on long-lived extracellular proteins whereas more recent studies have highlighted the importance of intracellular glycation. The chemical nature of many AGEs, their synthesis *in vivo* and their precise role in the pathogenesis of diabetic complications is under intense investigation (Nessar Ahmed, 2005). Glycation is a major cause of spontaneous damage to cellular and extracellular proteins in physiological systems, accounting for 0.1–0.2% of lysine and arginine residues (Thornalley, 2003). Reactive dicarbonyl compounds formed endogenously, glyoxal, methylglyoxal and 3-deoxyglucosone, are potent glycating agents. Glycation by methylglyoxal is increased disproportionately compared to the increase in glucose concentration in experimental and clinical diabetes (McLellan *et al.*, 1994). This may be attributed to the formation of methylglyoxal from triosephosphate accumulation in vascular cells suffering cytosolic hyperglycaemia (Brownlee, 2001).

Type I diabetes is due to a deficiency of insulin as a result of destruction of the pancreatic β cells. At the time of clinical symptoms, 60–80% of the β cells are destroyed. Cells secreting glucagon, somatostatin and pancreatic polypeptide are generally preserved but may be redistributed within the islets. For years, the notion that T-lymphocytes played a crucial role in disease induction was considered such a sound dogma that interest in B-lymphocytes and auto-antibodies as pathogenic variables was largely relegated to second-class status. However, much of our knowledge regarding the pathogenesis and natural history of this disease has been obtained by analysis of subjects having type I associated auto-antibodies. While auto-antibodies to more than two dozen auto-antigens have been associated with this

disease, a majority of interest has been directed to islet cell auto-antibodies (ICA), insulin auto-antibodies (IAA), glutamic acid decarboxylase (GAD) and tyrosine phosphatase-like IA-2 auto-antigen. These auto-antibodies, combined with other metabolic and genetic markers, are extremely effective for predicting eventual development of type I diabetes.

Like type I diabetes, type II diabetes also involves both genetic susceptibility and environmental factors, although the genetic component may be greater than in type I diabetes. It is caused by a combination of insulin resistance and relative insulin deficiency with increased hepatic glucose production. Insulin resistance alone, however, does not cause diabetes. Most obese people do not develop type II diabetes, despite increased insulin resistance (Polansky, 2000). Before type II diabetes develops, the pancreatic β-cells increase their production of insulin to compensate for increased insulin resistance. For unclear reasons, β-cell secretory capacity gradually declines in some individuals, leading to the development of type II diabetes. As β-cell insulin secretory capacity declines, type II diabetes begins to develop. Initially, hyperglycemia is only observed after large meals, as in type I diabetes. As β-cell function declines further, hyperglycemia becomes more severe. Several authors speculate that increased insulin resistance may be a genetic trait that can be worsened by obesity and that β-cells compensate for this increased resistance. Some individuals, however, cannot maintain this compensation because of β-cell failure, which leads to the development of type II diabetes.

In view of this, the possible involvement of MG-Lys-Cu^{2+} modified human DNA in diabetes mellitus (both type I & II) was probed. Diabetes type I and II sera were screened for the presence of autoantibodies reactive to native and MG-Lys-Cu^{2+} modified human DNA. The binding of circulating autoantibodies from eighty five type I and II diabetes patients and twenty healthy normal subjects to native and MG-Lys-Cu^{2+} modified human DNA was studied by direct binding ELISA. Of the 40 sera in type I diabetes, 67.5% showed preferentially high binding to MG-Lys-Cu^{2+} modified human DNA as compared to its native analogue. No appreciable binding either with native or MG-Lys-Cu^{2+} modified human DNA was observed with serum antibodies from healthy normal subjects. Competition ELISA results showed 22 to 33% inhibition in the type-I diabetes autoantibodies binding to native human DNA,

whereas 46.9 to 63.1 % inhibition was observed with MG-Lys-Cu^{2+} modified human DNA. These results indicate appreciable recognition of MG-Lys-Cu^{2+} modified human DNA by the autoantibodies in diabetes (type-I) patients. The binding specificity of the isolated IgG, towards native and MG-Lys-Cu^{2+} modified human DNA was evaluated by competition ELISA. Immunoglobulin G (IgG) from diabetic patients (type-I) recorded an inhibition of 59% to 69% with the MG-Lys-Cu^{2+} modified human DNA, while with native human DNA it ranged from 27% to 36%. Appreciably high binding of affinity purified IgG towards MG-Lys-Cu^{2+} modified human DNA, is indicative of the generation of antibodies against RCS modified epitopes on the DNA molecules in diabetes.

The strong binding of autoantibodies from diabetes type I patients to MG-Lys-Cu^{2+} modified human DNA is evidence towards the involvement of modified bases and single strand regions in disease pathogenesis. The spontaneous production of auto-antibodies in type I diabetes might be a result of the generation of the antigenic epitopes on the DNA molecules that are recognized as 'non self' by the body's immune system. It could, therefore, be one of the factors of the immune response in diabetes.

In type II diabetes patients (total 45), 35.5% serum samples showed low to moderate binding to MG-Lys-Cu^{2+} modified human DNA as compared to its native analogue. The degree of binding was moderately higher than serum antibodies from normal healthy subjects. The moderate (average) recognition of MG-Lys-Cu^{2+} modified human DNA by the auto-antibodies in type II diabetes patients was further confirmed by competitive inhibition analysis. The inhibition in the antibody activity with MG-Lys-Cu^{2+} modified human DNA was in the range of 34.4% to 53%, however for native human DNA it was found to be 18% to 30.1%. These results are distinct from type I diabetes. In type II diabetes, the recognition of auto-antibodies against MG-Lys-Cu^{2+} modified human DNA is quite low as compared to type I diabetes. The IgG isolated from type II diabetes patients recorded an inhibition in the range of 24% to 36% and 49% to 57%, when MG-Lys-Cu^{2+} modified human DNA and its native form were respectively used as inhibitors. The results show ample evidence of the involvement of MG-Lys-Cu^{2+} modified human DNA in type I diabetes while the same is less established for the type II.

Based on above studies the following conclusions can be drawn-

1. MG-Lys-Cu^{2+} modification of human DNA results in the formation of strand breaks and base modification.
2. Fluorescence spectroscopy in combination with anti-glycation study (by D-penicillamine and pyridoxal phosphate) supports the formation of glycation products.
3. Comet assay shows the genotoxicity of advanced glycation end-products formed by MG-Lys-Cu^{2+} system on the human DNA.
4. Thermal denaturation studies show that modification has rendered the human DNA susceptible to rise in temperature. This shows the destabilization of helix.
5. Estimation of free radicals ($^{\cdot}OH$ and $O_2^{\cdot-}$) and their quenching studies in the presence of specific quenchers confirms the formation of free radicals in MG-Lys and MG-Lys-Cu^{2+} system respectively.
6. Both HPLC and LC-MS supported the formation of the adduct, carboxy-ethyl deoxy-guanosine (CEdG) in modified human DNA. CEdG is a useful biomarker for monitoring oxoaldehyde-induced stress in response to enhanced glycolytic flux or environmental exposure to MG.
7. ESI-MS data supports the formation of Schiff base, Schiff base dimer and amadori product in glycated human DNA samples.
8. MG-Lys-Cu^{2+} modification of human DNA renders it highly immunogenic inducing high titre antibodies in experimental animals. This shows the generation of neo-epitopes on the DNA molecule upon modification.
9. The induced antibodies, though highly specific for the immunogen, also exhibited polyspecific binding. The antibodies showed binding with various nucleic acid conformers and nitrogenous bases significantly recognizing epitopes formed as a result of MG-Lys-Cu^{2+} modification.
10. Diabetes type I auto-antibodies showed preferential binding to MG-Lys-Cu^{2+} modified human DNA as compared to the native form. This shows that RCS modified DNA could be an antigenic stimulus for these auto-antibody.
11. Antibodies from type II diabetes patients exhibited low to moderate binding with MG-Lys-Cu^{2+} modified human DNA. This means that some type II diabetes patients (especially with secondary complications) develop antibodies against RCS modified DNA.

The RCS system used in the study has direct physiological relevance as MG is formed endogenously and is enhanced 5-6 times in pathogenesis of diabetes and diabetic complications. Moreover, lysine is an essential amino acid which is present in most proteins and also a part of enzyme active site. It is also the preferred group for non enzymatic carbonyl group attachment and subsequent formation of AGEs. Methylglyoxal can readily react with lysine to form AGEs and generates free radicals. The generation of free radicals is enhanced in the presence of cu^{2+}, which is present *in vivo* in variety of enzymes like cytochrome c oxidase and superoxide dismutases. Thus MG-lys-Cu^{2+} system can be very deleterious for macromolecules like DNA, proteins, lipids etc. The present study has probed the MG-lys-Cu^{2+} induced damage to DNA and the resulting immune response of the modified DNA and its role in the induction of antibody response in diabetes.

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